University of Alberta

Characterization of a novel periplasmic accessory protein, CpxP

by

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Abstract

The *Escherichia coli* Cpx two component signal transduction (TCST) system uses a phosphotransfer event to propagate a cellular response to stresses that involve misfolded proteins that are associated with the periplasmic face of the inner membrane. It consists of a histidine kinase, CpxA and a response regulator, CpxR. An interesting aspect of the Cpx pathway is the presence of a third component, CpxP. CpxP is a periplasmic accessory factor that was initially characterized as an inhibitor protein. Given that CpxP has no informative homologues, little was known about the role CpxP plays in the signal transduction of the Cpx pathway. Using a translational fusion protein, CpxP-β-lactamase (CpxP'-'Bla) and random mutagenesis, a highly conserved N-terminal domain within CpxP that is important for Cpx pathway inhibition and a C-terminal domain that is implicated in protein stability were identified. Additionally, CpxP'-'Bla was shown to be degraded by the periplasmic protease DegP in response to alkaline pH. CpxP'-'Bla half life analysis revealed that CpxP stability depended on the inducing cue present, suggesting that there are at least two ways in which the Cpx pathway can be activated. Epistasis experiments showed that CpxP and DegP are important for proper activation of the Cpx response and that DegP functions upstream of CpxP in the signaling cascade. Furthermore, CpxP is needed to efficiently shut off of the Cpx response. Structural studies were initiated to gain insight into the roles CpxP plays in signal transduction and protein folding. *In vivo* cross-linking, circular dichroism, Small angle x-ray analysis, and size exclusion chromatography showed that CpxP is an α-helical dimer both *in vivo* and *in vitro*, that undergoes a slight conformational change in response to alkaline pH.

Finally, a novel transcriptional regulator, YifE, was identified that influences Cpx gene expression independently of CpxRA. YifE was shown to be a global regulator that may respond to cytoplasmic misfolded proteins via the chaperone, DnaK.

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Table of Contents

1.0 Introduction. 1

1.1 Introduction. 2

1.2 Bacterial Envelope. 2

1.3 Envelope stress response. 3

1.4 The σ^E stress response. 4

1.4.1 What does σ^E sense? 4

1.4.2 How is σ^E regulated? 5

1.4.3 What role does the σ^E play in the cell? 8

1.5 Two component signal transduction. 10

1.5.1 How do histidine kinases sense their surroundings? 11

1.5.2 The role of accessory proteins in regulation of two component signal transduction. 14

1.5.3 How do response regulators regulate gene expression? 17

1.6 The Cpx response. 18

1.6.1 Discovery of the Cpx pathway and its components. 18

1.6.2 Activating cues of the Cpx pathway. 19

1.6.3 Inhibition of the Cpx pathway. 21

1.6.4 The Cpx regulon helps alleviate envelope stress. 22

1.6.4.1 The role of DegP in alleviating envelope stress. 23

1.6.5 The role of the Cpx response. 24

1.7 Proposed research project. 25

1.7.1 Characterization of the periplasmic inhibitor protein, CpxP. 26

1.7.1.1 Identification of functional domains in CpxP that are important for inhibition. 26

1.7.1.2 Determining if CpxP and CpxA interact. 27

1.7.1.3 Dimerization of CpxP. 27

1.7.1.4 Determining a physiologically relevant role for CpxP. 27

1.7.2 Screen for additional regulators of the Cpx pathway. 28

1.8 References. 33

2.0 Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP. 45

2.1 Introduction. 46

2.2 Materials and methods. 48

2.2.1 Bacterial strains and plasmids. 48

2.2.2 Media, antibiotics and growth conditions. 48

2.2.3 Mutagenesis of pPB. 48

2.2.4 DNA sequence analysis. 48

2.2.5 β-galactosidase assay. 49

2.2.6 Western blot analysis. 49

2.2.7 Site-directed mutagenesis (SDM) of pCpxPU to make pCpxPD_{61}EU and pCpxPQ_{55}PU. 49

2.3 Results. 50

2.3.1 Inhibition of the Cpx pathway is specific to CpxP overexpression. 50

2.3.2 Isolation of *cpxP* mutants. 51

2.3.3 *cpxP* loss-of-function mutations localize to two distinct regions. 51

2.3.4 Loss-of-function CpxP mutants fail to inhibit the Cpx pathway. 52

2.3.5 Mutant CpxP'-'Bla proteins are expressed at varied levels. 52

2.3.6 Diminished mutant CpxP'-'Bla protein levels are restored by *degP* mutation. 53

2.3.7 Cpx pathway activity and CpxP'-'Bla levels are affected by DegP in response to elevated pH. 53

2.4 Discussion. 54

2.4.1 The Cpx envelope stress response is uniquely inhibited by CpxP overexpression. 55

2.4.2 A predicted N-terminal α-helix plays an essential role in CpxP-mediated inhibition and stability. 55

2.4.3 DegP influences both Cpx pathway activity and CpxP'-'Bla levels in response to inducing cues. 56

2.5 References. 68

3.0 The novel signaling protein CpxP is an α-helical dimer that functions with the protease DegP to fine-tune activation and shut-off of the Cpx envelope stress response in *Escherichia coli*. 71

3.1 Introduction. 72

3.2 Materials and Methods. 74

3.2.1 Bacterial strains and plasmids. 74

3.2.2 Media, antibiotics and growth conditions. 75

3.2.3 Western blot analysis. 75

3.2.4 Adsorption of non-specific antibodies from anti-CpxP serum. 75

3.2.5 Protein stability assays. 76

3.2.6 Construction of *cpxP* and *degP* mutant strains. 77

3.2.7 Monitoring Cpx pathway turn-on and shut-off using a *cpxP'-lux*$^+$ reporter. 77

3.2.8 Formaldehyde mediated in vivo cross-linking. 78

3.2.9 Expression and purification of wild type CpxP for biochemical assays. 78

3.2.10 Size-exclusion chromatography with Multi-angle Laser Light Scattering (MALLS). 79

3.2.11 Small angle X-ray analysis (SAXS). 80

3.2.12 Circular dichrosim. 80

3.3 Results. 81

3.3.1 CpxP'-'Bla stability is affected differently by DegP depending on the inducing cue. 81

3.3.2 CpxP and DegP are required for the rapid induction and shut-off of the Cpx response and DegP functions upstream of CpxP. 83

3.3.3 CpxP is an α-helical dimer that changes conformation, but is not misfolded, in the presence of the inducing cue, alkaline pH. 85

3.3.4 CpxP LOF mutants exhibit proteolysis and dimerization phenotypes. 87

3.4 Discussion. 89

3.4.1 Inducing cues differentially affect CpxP stability. 90

3.4.2 New CpxP signaling phenotypes. 90

3.4.3 Low resolution structure analysis of CpxP refines the model for its role in Cpx signal-transduction. 92

3.4.4 CpxP is an α-helical dimer. 92

3.4.5 CpxP LOF mutants alter dimerization and DegP-dependent proteolysis. 93

3.5 References. 107

4.0 The highly conserved, uncharacterized transcription factor YifE and other cytoplasmic factors influence Cpx regulon expression independently of CpxRA. 112

4.1 Introduction. 113

4.2 Materials and methods. 115

4.2.1 Bacterial strains. 115

4.2.2 Media, antibiotics and growth conditions. 115

4.2.3 Transposon mutagenesis screen for regulators of *cpxP* expression. 116

4.2.4 Sequencing of the *yifE::*Tn10-*cam* and λp*lac*Mu53 insertion points. 116

4.2.5 Construction of *relA*::kan, *dnaK*::kan, *dksA*::kan and *yifE*::kan mutants. 117

4.2.6 β-galactosidase assay. 117

4.2.7 λp*lac*Mu53 infection. 117

4.3 Results. 118

4.3.1 A screen for factors affecting *cpxP'-lacZ*$^+$ expression. 118

4.3.2 *yifE*::Tn10-*cam* is a gain-of-function mutation. 118

4.3.3 YifE and the CpxRA two component system function independently to control expression of Cpx-regulated genes. 119

4.3.4 YifE regulates other envelope stress responses and is a global regulator of gene expression. 120

4.3.5 Expression of Cpx regulon genes is effected by other cytoplasmic regulators in addition to YifE. 121

4.3.6 DnaK is epistatic to YifE. 122

4.4 Discussion. 123

4.4.1 YifE influences expression of envelope stress genes. 123

4.4.2 A function for the highly conserved, uncharacterized protein, YifE. 124

4.4.3 Numerous cytoplasmic signals influence regulation of genes controlled by the Cpx response. 125

4.5 References. 136

5.0 Conclusions and Future Directions. 140

5.1 A highly conserved predicted N-terminal α-helix is important for CpxP inhibitory activity. 141

5.2 DegP mediates changes in CpxP stability differentially in response to inducing cues. 141

5.3 CpxP is an α-helical dimer that undergoes a conformational change at alkaline pH. 142

5.4 CpxP functions in both the inhibition and activation of the Cpx response. 143

5.5 A model for the role of CpxP in Cpx signal transduction. 144

5.6 The expression of the Cpx regulon is influenced not only by envelope stress but also cytoplasmic stresses and factors. 145

5.7 Questions that remain. 146

5.7.1 Does CpxP inhibit DegP activity? 146

5.7.2 Where is the DegP cleavage site in CpxP? 147

5.7.3 Is there a difference between the CpxP dimerization and inhibition domain? 148

5.7.4 Do CpxP and CpxA interact? 149

5.8 References. 160

Appendix A Characterization of a truncated version of CpxP 162

A.1 Introduction 163

A.2 Materials and Methods 163

A.2.1 Bacterial strains and plasmids 163

A.2.2 Construction of $pMCP_{40-151}$ and $pMCP_{20-151}$ 163

A.3 Results 164

A.3.1 Cpx pathway activity in the presence of $pMCP_{40-151}$ and $pMCP_{20-151}$ 164

A.3.2 $MBP-CpxP_{40-151}$ has similar expression levels compared to MBP-CpxP, while $MBP-CpxP_{20-151}$ is unstable 164

A.4 Conclusions 165

A.5. References 170

List of Tables

Table 2-1	Bacterial strains used in chapter 2.	59
Table 2-2	Plasmids used in chapter 2.	60
Table 2-3	Primers used for PCR in chapter 2.	61
Table 3-1	Bacterial strains used in chapter 3.	96
Table 3-2	Plasmids used in chapter 3.	97
Table 3-3	Half-life analysis of CpxP'-'Bla in response to Cpx inducing cues.	98
Table 4-1	Strains and plasmids used in chapter 4.	127
Table 4-2	Primers used for PCR in chapter 4.	128
Table 4-3	Random λ*plac*Mu53 insertions and envelope stress genes that exhibit regulation by *yifE*	129
Table 5-1	Bacterial strains used in chapter 5.	151
Table 5-2	Plasmids used in chapter 5.	152
Table A-1	Bacterial strains and plasmids used in Appendix A.	166
Table A-2	The effects on Cpx pathway activity when MBP-CpxP$_{20\text{-}151}$ and MBP-CpxP$_{40\text{-}151}$ are overexpressed.	167

List of Figures

Figure 1-1 Regulated intramembrane proteolysis of the σ^E pathway. 30

Figure 1-2 The full length sequence of CpxP. 31

Figure 1-3 Regulation of the Cpx pathway. 32

Figure 2-1 Inhibition of the Cpx response is specific to CpxP overexpression. 62

Figure 2-2 The effects of overexpression of LOF CpxP mutants on the Cpx pathway. 63

Figure 2-3 Expression levels of wild type and mutant CpxP'-'Bla fusion proteins in wild type and *degP* null strain backgrounds. 64

Figure 2-4 Induction of the Cpx pathway and CpxP'-'Bla levels in response to alkaline pH. 65

Figure 2-5 Sequence alignment of CpxP proteins from various bacterial species. 66

Figure 2-6 Model for activation of the Cpx pathway. 67

Figure 3-1 CpxP is involved in the activation and inhibition of the Cpx pathway. 99

Figure 3-2 CpxP and DegP influence Cpx pathway activity and DegP functions upstream of CpxP. 100

Figure 3-3 CpxP forms a dimer *in vivo*. 101

Figure 3-4 CpxP is a dimer at pH 5.8 and 8.0 but has slightly different conformations. 102

Figure 3-5 CpxP is mostly α-helical and slightly changes conformation but is not misfolded in response to the inducing cue of alkaline pH. 103

Figure 3-6 Inhibitory activity can be returned to the C-terminal Class 2 LOF CpxP mutant, CpxPQ$_{128}$H'-'Bla in a *degP* mutant background. 104

Figure 3-7 Characterization of CpxP LOF mutants. 105

Figure 3-8 Model for activation of the Cpx pathway. 106

Figure 4-1 *yifE*::Tn10-*cam* is a gain of function mutation. 130

Figure 4-2 YifE regulates Cpx regulon genes independently of CpxRA. 131

Figure 4-3 YifE affects the regulation of the σ^E-regulated *rpoHP3* promoter. 132

Figure 4-4 Expression of Cpx regulated genes is influenced by other cytoplasmic factors in addition to YifE. 133

Figure 4-5 DnaK affects expression of Cpx regulon members. 134

Figure 4-6 Model of how YifE inhibits expression of Cpx regulon genes. 135

Figure 5-1 Helical wheel analysis of the predicted N-terminal α-helical domain of CpxP that is important for inhibition and stability. 153

Figure 5-2 Sequence alignment of CpxP orthologues. 154

Figure 5-3 Complete model of the Cpx response. 155

Figure 5-4 Strategy and predictions of the titration assay between CpxP and GST-CpxA. 156

Figure 5-5 Activity of the Cpx pathway in the presence of an additional CpxA sensing domain and overexpression of CpxP'-'Bla in a wild type or *cpxA*::kan background. 157

Figure 5-6 Predicted secondary structure and topology of the sensing domain of CpxA. 158

Figure 5-7 Bacterial two-hybrid analysis of CpxP-CpxP dimerization. 159

Figure A-1 Sequence and schematic representation of truncated versions of CpxP. 168

Figure A-2 Expression levels of wild type and truncated versions of MBP-CpxP. 169

List of Abbreviations

A	alanine
aa	amino acid
Amik	amikacin
Amp	ampicillin
AMPs	antimicrobial peptides
AP	alkaline phosphatase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BFP	bundle forming pili
Bla	β-lactamase
Cam	chloramphenicol
cAMP	cyclic adenosine monophosphate
°C	degrees Celcius
CD	circular dichroism
CP	cytoplasm
D	aspartic acid / aspartate
D_{max}	maximum particle dimension
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	glutamic acid
ECF	extracytoplasmic function
EPEC	enteropathogenic *Escherichia coli*
ER	endoplasmic reticulum
F	phenylalanine
FLP	flippase
FRT	FLP recombinase target
G	glycine
GST	glutathione S-transferase
H	histidine
h	hours
HK	histidine kinase
I	isoleucine
IM	inner membrane
IPTG	isopropyl-β-D-galactoside

Kan	kanamycin
kDa	kilodalton
kb	kilobase
Lac	lactose
LB	Luria-Bertani
LD_{50}	50% lethal dose
LOF	loss of function
LPS	lipopolysaccharide
M	molar
mM	millimolar
MBP	maltose binding protein
min	minute
mL	milliliter
mRNA	messenger RNA
MW	molecular weight
m/z	mass to charge ratio
N	asparagines
nm	nanometer
N.S.D.	normalized standard deviations
O.D.	optical density
OM	outer membrane
OMP	outer membrane proteins
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PAS	Per, ARNT, and Sim
PCR	polymerase chain reaction
PG	peptidoglycan
ppGpp	3',5'-bispyrophosphate
PP	periplasm
PPI	peptodylproyl *cis*, *trans* isomerase
PMF	proton motive force
Rg	radius of gyration
R_H	radius of hydration
RNA	ribonucleic acid
RNAP	RNA polymerase
RR	response regulator

S	serine
SAXS	Small-angle X-ray scattering
SDS	sodium dodecyl sulfate
SDM	site directed mutagenesis
sec	second
Sm	streptomycin
Spc	spectinomycin
sRNA	small non-coding RNA
TCST	two component signal transduction
Tet	tetracycline
T_m	melting temperature
TTS	type III secretion
µg	microgram
µL	microliter
µm	micrometer
UMP	uracil monophosphate
UPEC	uropathogenic *E.coli*
UPR	unfolded protein response
UV	ultra violet
V	valine
W	tryptophan
W/W	by weight
x	any amino acid
Xaa	any amino acid
Y	tyrosine

Chapter 1:
Introduction

1.1 Introduction.

A microbe's existence can be characterized by its ability to survive interactions with other microbes and stresses in its surroundings. Some of these stresses include changes in osmotic activity, ionic strength, concentration of nutrients, pH, temperature and toxic compounds such as detergents, solvents or antibiotics. In Gram negative bacteria responses to such stresses can be compartmentalized into cytoplasmic and extracytoplasmic stress responses. The extracytoplasmic stress response, also known as the envelope stress response, monitors the integrity of the envelope.

1.2 Bacterial envelope.

The Gram negative bacterial envelope consists of the outer membrane (OM), inner membrane (IM) and the periplasm. The OM is an atypical bilayer that consists mainly of phospholipids, lipopolysaccharides (LPS), and proteins (Nikaido & Vaara, 1987). The composition of phospholipids in the OM is very similar to the IM however there is an enrichment of phosphatidylethanolamine (Nikaido & Vaara, 1987), which is mainly found in the inner leaflet of the OM. The LPS is a unique aspect of the OM and is found on the outer most leaflet. It is quite negatively charged and is strongly bound by divalent cations, which stabilize the physical structure of the LPS (Schindler & Osborn, 1979). OM proteins make up about half of the OM mass, with most of these proteins found exclusively in the OM. Some examples of OM proteins are lipoproteins, porins, specific channels, and high-affinity receptors (Nikaido & Vaara, 1987). The structure of the OM helps it carry out its many functions, such as resistance to host defense factors like lysozyme, various leukocyte proteins, bile salts and digestive enzymes. This is especially important for bacteria that live in the intestine (Nikaido & Vaara, 1987). The hydrophobilicity of the OM helps Gram negative bacteria evade phagocytosis, while the LPS lowers the permeability to lipophilic solutes and specific channels/porins help regulate the diffusion rate of nutrient transport (Nikaido & Vaara, 1987).

The IM is composed mainly of phospholipids and proteins that have restricted vertical travel but are free to diffuse within the plane of the membrane. The three main phospholipid species in the IM are phosphatidyethanolamine, phosphatidylglycerol, and caridiolipin, allowing it to act as a hydrophobic barrier that prevents uncontrolled movement of polar molecules while at the same time allowing for the accumulation and retention of metabolites/proteins (Cronan *et al.*, 1987). The IM carries out numerous cellular functions including energy generation and conservation, regulated transport of nutrients/metabolic products, translocation of envelope macromolecules and transmembrane signaling (Cronan *et al.*, 1987).

The periplasm lies between the OM and IM, with an estimated width of 13 to 25 nm, and contains the peptidoglycan layer, which is involved in cell shape. Originally, it was thought that the lateral diffusion rate of proteins in the periplasm was 1000 fold lower than the comparable aqueous medium and 100 fold lower then the cytoplasmic diffusion rate because of the high protein content and the unpolymerized peptidoglycan. However, recently it was shown that the diffusion rate of the periplasm was not much different than the cytoplasm, with an average diffusion rate of 9.0 ± 2.1 $\mu m^2 s^{-1}$ in the cytoplasm and 2.6 ± 1.2 $\mu m^2 s^{-1}$ in the periplasm (Mullineaux *et al.*, 2006). The periplasm is involved in processing essential nutrients for transport, biogenesis of major envelope components, detoxification, and buffering the cytoplasmic environment from external stresses to maintain growth and viability (Oliver, 1987). An interesting aspect of the periplasm is that it carries out all these functions in the absence of ATP.

1.3 Envelope stress responses.

Escherichia coli has at least six known envelope stress responses that help it monitor the integrity of the envelope. These stress responses include the Cpx (conjugative pilus expression), Bae (bacterial adaptive response), σ^E, Rcs (regulator of capsular synthesis), Psp (phage-shock-protein) and outer membrane vesicle responses. Of these six responses, three of them are regulated by two component signal transduction pathways, suggesting that two component systems play a critical role in maintaining envelope homeostasis. Although all six of these responses monitor envelope integrity, there is little overlap between the pathways, meaning that the majority of them sense and respond to different stresses. The Cpx pathway monitors and reacts mainly to stresses that involve misfolded and mislocalized proteins in the periplasm, specifically those associated with the outer leaflet of the inner membrane (Raivio & Silhavy, 2001). It has also been shown to sense and respond to adhesion to abiotic surfaces (Otto & Silhavy, 2002). The Bae signal transduction pathway is believed to be involved in ridding the cell of toxic compounds that may or may not damage the envelope (Garbe *et al.*, 2000, Baranova & Nikaido, 2002, Raffa & Raivio, 2002, Zhou *et al.*, 2003). The σ^E stress response monitors and responds to stresses that are involved in OM protein (OMP) folding and maintenance (Alba & Gross, 2004, Mecsas *et al.*, 1993, Erickson & Gross, 1989). The Rcs pathway senses disruptions to the peptidoglycan layer and helps in the formation of capsules (Majdalani & Gottesman, 2005, Huang *et al.*, 2006). The Psp response is involved in sensing and maintaining the proton motive force (PMF) (Darwin, 2005). Finally the outer membrane vesicle release response is a protective mechanism that helps alleviate the accumulation of misfolded proteins in the periplasm (McBroom & Kuehn, 2007). For the purpose of this thesis, I will only be discussing the Cpx two component system and the σ^E stress response in more detail.

1.4 The σ^E envelope stress response.

The σ^E response was the first envelope stress response described in the literature over 15 years ago (Erickson & Gross, 1989), therefore it is probably the best characterized envelope stress response to date. Having such a depth of knowledge of the σ^E pathway allows researchers to compare similarities between it and other envelope stress responses. It also allows one to speculate as to what is happening in these other stress responses. Since then, the σ^E response has been shown to play an important role in maintaining OMP homeostasis and also to be conserved in a number of microbes (Rhodius *et al.*, 2006).

1.4.1 What does σ^E sense?

σ^E falls into a class of ECF (extracytoplasmic function) sigma factors, which are divergent from σ^{70} and are used by bacteria to monitor their surroundings. It was first discovered by Erickson and Gross (1989) for its ability to regulate *rpoH* (heat shock σ factor) and *degP* (periplasmic serine endoprotease/chaperone) expression under heat shock conditions, which is the upshift in temperature above 50°C. This suggested that σ^E may regulate a stress response involved in protecting the envelope. To test this theory Mecsas *et al.* (1993), looked at what happened to σ^E in response to OMPs, such as OmpT, C, F, and X, when they were either limited or overexpressed. They showed that σ^E activity was increased in response to overexpression of OMPs, however when OMPs were limited σ^E activity was decreased (Mecsas *et al.*, 1993). This suggested that overexpression of OMPs induced σ^E, but it needed to be determined if that signal was generated in the cytoplasm or the periplasm. Mecsas *et al.* (1993) looked at what happened to σ^E activity when OMPs were overexpressed in a *secB* mutant, causing the OMPs to be trapped in the cytoplasm. The activity of the σ^E pathway in this background matched that seen when

OMPs were limited, confirming that σ^E is sensing the accumulation of OMPs in the periplasm (Mecsas *et al.*, 1993).

What OMP specific signal was generated upon OMP overexpression that induced the σ^E pathway? In the years to come it was discovered that effectors such as ethanol, DTT (dithiothreitol), and puromycin, which all cause misfolded proteins, also induced the σ^E pathway (Raina *et al.*, 1995). It was also shown that when the UPEC (uropathogenic *E.coli*) P-pili subunit, PapG, was overexpressed in the absence of its cognate periplasmic chaperone PapD, σ^E activity increased (Jones *et al.*, 1997). All these observations suggested that σ^E was responding to increased misfolded proteins in the envelope. To verify these results, Raina *et al.* (1995) and Missiakas *et al.* (1996) made mutations to protein folding genes, including *dsb* (disulfide bond oxidoreductase) genes, *degP*, *surA* (peptidylproyl *cis,trans* isomerase (PPI)/ chaperone), and *fkpA* (PPI/chaperone), and then measured σ^E activity, and found it to be increased (Raina *et al.*, 1995, Missiakas *et al.*, 1996). This verified that σ^E was sensing and responding to envelope stress caused by misfolded proteins, more specifically by misfolded OMPs.

1.4.2. How is σ^E regulated?

When σ^E was discovered, the σ factor responsible for upregulating *rpoH* and *degP* transcription was purified (Erickson & Gross, 1989), however the gene encoding σ^E was not known. Rouviere *et al.* (1995) and Raina *et al.* (1995) set out to identify the gene encoding σ^E. When analyzing and cloning σ^E, they noticed that in the absence of induction its activity was low, indicating to them that it was negatively regulated (Rouviere *et al.*, 1995, Raina *et al.*, 1995). To try and identify a possible anti-sigma factor that was responsible for this negative regulation two different approaches were taken; the first relied on the fact that normally sigma factors and anti-sigma factors are found in the same operon (De Las Penas *et al.*, 1997a), the second approach used transposon mutagenesis to look for genes that, when disrupted, lead to an increase in σ^E activity (Missiakas *et al.*, 1997). Both approaches identified the first two genes found downstream of σ^E in the *rpoErseABC* operon (Missiakas *et al.*, 1997, De Las Penas *et al.*, 1997a). It was determined that RseA (regulator of sigma E) was an inner membrane protein that is the central regulatory molecule needed to transmit the signal from the envelope to σ^E and RseB is a periplasmic protein that is dispensable for transmitting the signal to σ^E but is still a negative regulator (De Las Penas *et al.*, 1997a, Missiakas *et al.*, 1997, Collinet *et al.*, 2000).

Much work has gone into characterizing these two proteins with respect to what role they play in response to stresses and how they regulate σ^E. As mentioned before RseA is an inner membrane protein that spans the membrane once with the C-terminus in the periplasm and the N-terminus in the cytoplasm (Missiakas *et al.*, 1997). It is the N-terminus that interacts with σ^E to inhibit it and is all that is needed for inhibition (Missiakas *et al.*, 1997, Campbell *et al.*, 2003, Tam *et al.*, 2002)(Figure 1-1). Originally it was shown that RseA interacts with σ^E, by binding to region 4.2, which shows high homology to the σ^{70} 4.2 region (Tam *et al.*, 2002). The σ^{70} 4.2 region is responsible for promoter recognition, more specifically in binding to the -35 region of the promoter (Severinova *et al.*, 1996). This suggests that the interaction between RseA and σ^E blocks promoter recoginition (Tam *et al.*, 2002). The crystal structure of the complex between RseA and σ^E, allowed for a better understanding of how RseA inhibited σ^E activity. The crystal structure showed that RseA was sandwiched between region 2 and region 4 of σ^E (Campbell *et al.*, 2003). As mentioned before region 4 is responsible for promoter recognition, while region 2 is responsible for binding the core RNAP and promoter recognition (Severinova *et al.*, 1996). Thus RseA inhibits σ^E through two mechanisms, one being sterically occluding σ^E from binding the core RNAP and the other blocking promoter recoginition. The role of RseB in this inhibition is in fine-tuning the response. RseB interacts with the C-terminal domain of RseA, from positions 160 to 189 (Cezairliyan & Sauer, 2007)(Figure 1-1). This interaction between RseB and RseA stabilizes the interaction between RseA and σ^E (De Las Penas *et al.*, 1997a, Missiakas *et al.*, 1997, Collinet *et al.*, 2000, Ades *et al.*, 1999). When RseB is absent there is a two-fold increase in σ^E activity compared to when it is present and the RseA half-life is changed from 18.6 ± 3.1 mins to 44.3 ± 6.1 mins, respectively (Ades *et al.*, 2003, Missiakas *et al.*, 1997, De Las Penas *et al.*, 1997a).

The increased stability of RseA when RseB is present (Collinet *et al.*, 2000, Ades *et al.*, 2003), the observation that upon stress RseA levels initially drop 2.5 fold even though the relative synthesis rate is the same, and the change in RseA half life from 44.3 ± 6.1 mins to 5.2 ± 2.7 mins upon induction suggests that RseA is post-translationally modified in response to stress (Ades *et al.*, 2003, Ades *et al.*, 1999). RseA is degraded sequentially by two inner membrane proteases, DegS (serine endoprotease) and RseP (YaeL)(zinc metalloprotease) upon induction of the σ^E response (Ades *et al.*, 1999, Kanehara *et al.*, 2002)(Figure 1-1). Thus the σ^E pathway is regulated by intramembrane proteolysis (Brown *et al.*, 2000).

It was initially thought that RseA was the sensing protein that transmitted the signal to σ^E when misfolded OMPs are present, but in actual fact it is the PDZ domain of DegS that senses misfolded OMPs by the YFF peptide found in the C-terminus of OMPs (Walsh *et al.*, 2003). The binding of OMP to the PDZ domain is thought to relieve the inhibition of the PDZ domain on the protease domain of DegS, allowing it to cleave RseA (Walsh *et al.*, 2003). The PDZ domain of DegS is not essential for cleavage of RseA, suggesting that the protease domain of DegS is responsible for recognizing the RseA cleavage site and cleaving RseA (Cezairliyan & Sauer, 2007, Walsh *et al.*, 2003). Under inducing conditions, misfolded OMPs bind to DegS, which in turn cleaves RseA on the periplasmic side of the transmembrane domain between V^{148} and S^{149} (Walsh *et al.*, 2003). This cleavage of RseA by DegS is the rate determining step and also serves to produce the substrate for RseP cleavage (Kanehara *et al.*, 2002, Ades *et al.*, 1999, Alba *et al.*, 2002). RseP cleaves the truncated RseA, which lacks the periplasmic domain, on the cytoplasmic side of the transmembrane domain at the cleavage site HExxH (Kanehara *et al.*, 2003). It is thought that the glutamine rich sequence of the periplasmic domain of RseA interacts with the periplasmic PDZ domain of RseP, inhibiting it until the appropriate signal is sensed (Kanehara *et al.*, 2003).

It has recently been shown that RseB plays a role in the proteolysis of RseA. As mentioned before RseB stabilizes RseA and therefore increases the stability of the complex of RseA and σ^E (Collinet *et al.*, 2000, Ades *et al.*, 2003, Missiakas *et al.*, 1997, De Las Penas *et al.*, 1997a), but the mechanism by which RseB did this was a major question that was only answered recently. Cezairliyan and Sauer (2007) showed *in vitro* that cleavage of RseA by DegS is inhibited by RseB even in the presence of misfolded OMPs. It was thought that this may be because RseB blocks the DegS cleavage site in RseA. However, the RseB binding site, as mentioned before, is between aa 160-189 of RseA, and the DegS cleavage site is between V^{148} and S^{149}, so RseB binding to RseA does not block the DegS cleavage site but it does makes RseA a poor substrate for DegS, by shielding DegS from accessing the cleavage site (Cezairliyan & Sauer, 2007). The RseB shielding of RseA is independent of the DegS PDZ domain and OMPs, and thus this shielding needs to be inactivated before RseA can be cleaved (Cezairliyan & Sauer, 2007), which suggests that there may be additional signals that RseB recognizes under stressful conditions. Not only does RseB seem to shield DegS cleavage of RseA but is also seems to diminish RseP degradation of RseA (Grigorova *et al.*, 2004)(Figure 1-1). So it now seems that RseB has a role in inhibition of both RseA cleavage events. Exactly when RseB is physiologically relevant remains to be answered, however one could suggest that it plays an important role in blocking the σ^E pathway from normal OMP misfolding "noise". While another suggestion may be that RseB is involved in fine tuning the response after the stress has been relieved, stopping both DegS and RseP from cleaving any newly synthesized RseA. In agreement with this idea, Ades *et al.* (2003) showed that any newly synthesized RseA during shut off is resistant to degradation, with a half life of over 50 minutes compared to 7.8 ± 0.8 mins when there is no stress present.

After the cleavage event by DegS and RseP, the cytoplasmic domain of RseA remains bound to σ^E (Figure 1-1), which is still able to inhibit σ^E from binding to RNAP, suggesting an additional cleavage event is needed to free σ^E from RseA. Once RseA is cleaved by RseP, a C-terminal VAA signal is produced that SspB, a ClpXP adaptor protein, recognizes (Flynn *et al.*, 2004). SspB binds the cytoplasmic domain of RseA in complex with σ^E and delivers it to ClpXP, where the remainder of RseA is degraded, freeing σ^E (Figure 1-1)(Flynn *et al.*, 2004). σ^E is now able to bind to RNAP and up regulate its regulon (Figure 1-1).

From the first discovery of how σ^E was regulated it was thought that the pivotal protein in signal transduction was RseA. But recently it was shown that σ^E activity increases during stationary phase independently of RseA (Costanzo & Ades, 2006). The increased activity of σ^E was shown to be because of a general cytoplasmic starvation signal. Further analysis showed that increased σ^E activity was correlated with an accumulation of ppGpp (3',5'-bispyrophosphate) levels (Costanzo & Ades, 2006). ppGpp is a global regulator for the general starvation signal and has also been shown to regulate σ^S and σ^N (Magnusson *et al.*, 2005). The observation that σ^E, σ^S, and σ^N are all regulated by ppGpp, suggests that this signal generates a broad response to nutritional stress. The mechanism by which ppGpp activates σ^E is not understood. However it is the first time a cytoplasmic signal has been shown to influence σ^E activity. Insight into the mechanism of how ppGpp activates σ^E will open the door to understanding how envelope stress responses are regulated by both external and internal signals.

1.4.3 What role does the σ^E pathway play in the cell?

To understand the role σ^E plays within the cell, one not only needs to look at the σ^E regulon members and what induces the pathway but also what physiological affects an *rpoE* knockout has in *E.coli* as well as other Gram negative bacteria. The exact role that σ^E plays in *E. coli* has been hard to discern because not long after it was discovered it was determined that it was essential (Hiratsu *et al.*, 1995, De Las Penas *et al.*, 1997b). While σ^E is essential in *E.coli*, its homologues are not essential, which has allowed researchers to study σ^E in these organisms with a little more ease.

While the first clues as to a physiological role for σ^E came from learning what induced the pathway, analysis of the regulon members has given us more of an idea as to what it is doing in the cell. The first identified members of the σ^E regulon included periplasmic chaperones and protein folding and degradation factors. These members include *skp* (a chaperone of OMPs), *fkpA* (PPI/chaperone), *dsbC* (disulfide bond oxidoreductase), and *surA* (PPI/chaperone) (Dartigalongue *et al.*, 2001). Interestingly, FkpA, DsbC and SurA were identified for their ability to modulate activity of σ^E, prior to their identification as regulon members (Raina *et al.*, 1995, Missiakas *et al.*, 1996). Upon further characterization, the σ^E regulon was expanded to include members involved in many aspects of cellular function (Rhodius *et al.*, 2006). However, it does seem that the primary role of σ^E is in monitoring the envelope since many regulon members are involved in periplasmic protein folding and degradation, biogenesis and assembly of OMPs, as well as regulating small non-coding RNAs (sRNAs) that reduce OMP RNA levels (Dartigalongue *et al.*, 2001, Johansen *et al.*, 2006, Onufryk *et al.*, 2005, Rezuchova *et al.*, 2003, Missiakas *et al.*, 1996, Erickson & Gross, 1989).

While it was already known that σ^E alleviates OMP misfolding, it was thought that it did this by expressing protein folding and degrading factors, as well as OMP assembly factors, such as *yifO* (an essential lipoprotein required for OMP biogenesis), *yraP* (non-essential lipoprotein that is important for cell envelope maintenance), and *yaeT* (an essential OMP assembly factor) (Onufryk *et al.*, 2005, Ruiz *et al.*, 2005, Wu *et al.*, 2005). Along with controlling misfolded OMPs this way, it was recently shown that σ^E controls the expression of OMPs themselves. It was noticed that when σ^E was overexpressed many OMPs, such as OmpA, OmpC, OmpF, OmpW and OmpX were downregulated even though their promoters do not contain a σ^E recognition sequence (Rhodius *et al.*, 2006, Kabir *et al.*, 2005). It was discovered the upon induction of the σ^E pathway, σ^E upregulates two sRNAs, *micA* and *rybB* (Johansen *et al.*, 2006). When MicA and RybB are upregulated they bind to *ompA* mRNA and *ompC/W* mRNA, respectively, decreasing the steady-state levels of these mRNAs (Johansen *et al.*, 2006), which in turn stops the accumulation of misfolded/mislocalized OMPs. This mechanism allows the other protein folding and degrading factors upregulated by σ^E to clear the misfolded proteins present and return the envelope to homeostasis.

A similar mechanism was shown to operate in *rpoE* null cells bearing suppressor mutations. While σ^E in *E. coli* is essential, cells lacking σ^E will grow if they acquire a suppressor (De Las Penas *et al.*, 1997b). In order to understand the role of σ^E in the cell in further depth, Douchin *et al.* (2006) looked at how suppressors alleviated the need for RseP in the normal activation of σ^E. They determined that when a sRNA, *rseX*, is overexpressed, the requirement for RseP is bypassed (Douchin *et al.*, 2006). Further analysis showed RseX was able to down regulate the levels of OmpC and OmpA (Douchin *et al.*, 2006), thus decreasing the potential envelope stress caused by the presence of these proteins and ultimately changing the cells need for σ^E.

While it is rather clear that the role for σ^E in *E.coli* is to help maintain envelope homeostasis, homologues of σ^E in other Gram negative bacteria have linked it to virulence. An *rpoE* mutant in *Salmonella enterica* serovar Typhimurium showed decreased survival in macrophages and epithelial cell lines and had an increased sensitivity to reactive oxygen species such as H_2O_2 and superoxide (Humphreys *et al.*, 1999, Kenyon *et al.*, 2002, Testerman *et al.*, 2002). These phenotypes were common among other Gram negative pathogens. For example a *Vibrio cholerae* σ^E mutant had decreased survival in intestinal environments and had an LD_{50} (50% lethal dose) 3 fold higher than wild type (Kovacikova & Skorupski, 2002). *Mycobacterium rpoE* mutants were unable to survive in macrophages which resulted in a delayed death in infected mice (Ando *et al.*, 2003, Manganelli *et al.*, 2004). σ^E mutants in *Burkholderia pseudomallei* exhibited reduced survival in macrophages, increased sensitivity to H_2O_2, and reduced ability to form biofilms, which facilitate resistance to antibiotics and the host immune system (Korbsrisate *et al.*, 2005). Thus, it seems that the role of σ^E in various pathogens is to mediate adaptation to oxidative stress. Exactly how it accomplishes this adaptation is not known. One could speculate that since σ^E plays such a major role in monitoring the envelope in *E.coli*, that adaptation to oxidative stress in other Gram negative bacteria may involve the envelope.

1.5 Two component signal transduction.

The Cpx pathway is one of three two component systems that is used by *E. coli* to monitor the status of the envelope and then transduce the signal across the inner membrane. In *E.coli* alone there are 66 two component signal transduction (TCST) proteins, consisting of 29 histidine kinases (HK) and 37 response regulators (RR)(Koretke *et al.*, 2000). Thus TCST systems play a major role in many cellular responses to alterations in the environment and not just in monitoring the envelope. TCST systems are the most abundant in prokaryotes, with free-living species generally having more than pathogens, since their environments are more variable (Koretke *et al.*, 2000). Hoch and Silhavy (Hoch & Silhavy, 1995) used the analogy that if bacteria did not have any TCST systems the bacteria would be rendered the equivalent of deaf, dumb and blind.

It is thought that HK and RR coevolved from a common ancestor because when Nixon *et al.* (1986) compared the sequence of *ntrB* and *ntrC* from *Bradyrhizobium parasponiae* to other homologous regulatory protein pairs, such as *envZ/ompR*, and *phoRB*, they noticed that the C-terminal regions of the HKs all shared extensive homology, while the N-terminal regions of the RRs were conserved (Nixon *et al.*, 1986, Koretke *et al.*, 2000). It is hypothesized that coevolution was the result of a gene duplication, since most HK and RR pairs are arranged in an operon (Mizuno, 1997). The model that was set forth was that the nonconserved N-terminal domain of the HK perceives a signal. This signal is then transferred to the conserved C-terminal domain of the HK, which then either interacts with or modifies the conserved N-terminal domain of the RR, ultimately activating the C-terminal domain, which is then able to elicit the appropriate response (Nixon *et al.*, 1986). It was later shown that this model was true and the way the signal is transferred from the HK to the RR is by means of a phosphorylation reaction (Ninfa & Magasanik, 1986, Igo *et al.*, 1989). Ultimately it is the HK that specifically controls the level of phosphorylation of its cognate RR.

1.5.1 How do histidine kinases sense their surroundings?

The role of HK in TCST systems has been the subject of much research over the past couple of decades. While much is known about how the HK transfers the signal to the RR, little is known about how HKs sense their signals. The most common or orthodox HK consists of a sensing domain, kinase/transmitter domain, and a linker/HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis, and phosphatase) domain, which is responsible for transmitting the signal from the sensing domain to the kinase domain (Nixon *et al.*, 1986, Parkinson & Kofoid, 1992, Appleman & Stewart, 2003). The kinase domain, which has been divided into two subdomains, the catalytic and dimerization domains, has been the focus of much of the research. Although between different HKs the kinase domain only shares approximately 25% homology, there are conserved amino acid motifs/boxes, that have been designated the N, D, F, G1, G2, and H boxes (Stock *et al.*, 1995, Kim & Forst, 2001), that are extremely important for the catalytic activity of the kinase domain. The N, D, F, G1 and G2 motifs are thought to be arranged in the mature folded protein so that they form the nucleotide binding surface that binds ATP and Mg^{2+} once a signal has been perceived (Stock *et al.*, 1995). When this signal is perceived two HKs dimerize via a four helix bundle formed from the dimerization domains and cross phosphorylate each other on a conserved histidine residue within the H-box, which is found within the dimerization domain. The phosphate is then transferred to the cognate RR (Igo *et al.*, 1989). Not only do HKs have kinase activity to autophosphorylate but after the stress has been relieved they are able to dephosphorylate the cognate RR using a phosphatase activity (Igo *et al.*, 1989).

While the main way that HKs have been classified is based on conserved boxes in the catalytic domain (Grebe & Stock, 1999, Kim & Forst, 2001), this might not be the best way to classify them. A better way to classify them may be via the divergent sensing domains and any motifs that they might contain (Mascher *et al.*, 2006), since it is these domains that specify cellular role. Mascher *et al.* (2006) classified HKs based on how they sense and it was noticed that they could be grouped into HKs that use; 1) a sensing mechanism associated with the membrane-spanning helices, 2) cytoplasmic sensing, and 3) periplasmic/extracellular sensing. Although these are the three major classes, there can be combinations of different sensing mechanisms, which may allow the HKs to sense many different signals at a given time or it may allow integration of a signal using two different mechanisms to amplify the response (Mascher *et al.*, 2006).

HKs that use transmembrane sensing make up the smallest class of sensor kinases, and are characterized by the presence of 2 to 20 transmembrane helices that are connected by short periplasmic and cytoplasmic linkers (Mascher *et al.*, 2006). These HKs most commonly sense mechanical properties of the inner membrane, such as turgor and rigidity. However they are also involved in sensing stimuli that are involved in transport, compounds that affect inner membrane integrity, and ion/ electrochemical gradients (Mascher *et al.*, 2006).

Cytoplasmic sensing HKs make up the second largest group, and can either be soluble or membrane anchored, however, their sensing domain must by cytoplasmic. Given that their sensing domain is found within the cytoplasm, the stimuli for these HKs must be derived from the cytoplasm and is generally thought to be either the presence or absence of cytoplasmic solutes. Identification of membrane-bound cytoplasmic sensing HK have relied on the presence of conserved cytoplasmic PAS domains. The PAS domain is named after three proteins, Per (period circadian), ARNT (Ah receptor nuclear translocator), and Sim (single-minded), that contain this domain. PAS domains were first identified in eukaryotes, but now have been found in all taxonomic groupings, and linked to protein-protein interactions.

Periplasmic sensing HKs are the largest class according to this classification. They contain all the same domains as most HKs. However, the periplasmic sensing domain is flanked by two transmembrane helices. For periplasmic sensing HKs, the signal is only propagated to the kinase domain via the HAMP linker and one of the transmembrane helices. Exactly how these HKs sense activating signals is not well understood. The best model for how this class of HKs sense inducing cues is based on a ligand-binding mediated signal transduction model, where a ligand binds to the sensing domain of the HK, altering its enzymatic activity. There are two types of ligand-binding mediated sensing; direct and indirect (Raivio, 2006). Direct sensing occurs when the HK responds to stimuli through direct binding of the sensing domain. Indirect sensing occurs when the HK receives information (a stimulus) from a secondary source, such as an accessory protein/factor (Raivio, 2006).

Traditionally, ions are not thought to act as primary messengers in signal transduction pathways. They usually act as secondary messengers or cofactors in biochemical reactions. However, it has been shown that divalent cations, as well as anions, are able to act as ligands in direct sensing by HKs. *Salmonella spp*. have two periplasmic sensing TCST systems, PhoPQ and PmrAB, which respond to extracellular divalent cation levels by direct interaction of the ions with the periplasmic sensing domain of the HKs, which regulate virulence determinants (Chamnongpol *et al.*, 2002, Vescovi *et al.*, 1996, Groisman, 1998, Wosten *et al.*, 2000). When there is an abundance of Mg^{2+} or Ca^{2+}, the cations bind to the periplasmic domain of PhoQ, causing it to take on a phosphatase conformation, thus shutting off the pathway (Vescovi *et al.*, 1997, Vescovi *et al.*, 1996, Montagne *et al.*, 2001). PmrAB TCST responds to cellular levels of Fe^{3+}; when Fe^{3+} is high, it binds the sensing domain of PmrB, analogous to Mg^{2+} and PhoQ (Wosten *et al.*, 2000). The difference between PmrB and PhoQ is that when Fe^{3+} interacts with PmrB, it causes PmrB to take on a kinase conformation, activating the pathway, and ultimately upregulating virulence determinant expression (Wosten *et al.*, 2000). The CitAB TCST system of *Klebsiella pneumoniae* responds to citrate levels and is essential for induction of citrate fermentation genes (Bott, 1997, Bott *et al.*, 1995). It was demonstrated that CitA was able to recognize and bind H-citrate^{2-} (Kaspar *et al.*, 1999), showing that it is the binding of citrate to CitA that activates it, which in returns activates CitB, which then up regulates citrate fermentation genes.

The crystal structures of the water-soluble sensing domains for both CitA and PhoQ have been determined (Cho *et al.*, 2006, Reinelt *et al.*, 2003). As well, the NMR structure of the periplasmic sensing domain of DcuS, which belongs to the CitA family of HKs, has been determined (Pappalardo *et al.*, 2003). In all three cases the sensing domain contains a PAS domain. Originally it was thought that PAS domains were intracellular domains involved in protein-protein interactions and sensing oxygen, redox conditions and light (Taylor & Zhulin, 1999). However with the recent discovery of PAS domains in the periplasmic sensing domains of HKs, the role that PAS domains play in signaling now can be expanded to extracellular sensing. As well as the role that PAS domains play in extracellular sensing, they have also been implicated in protein-ligand binding instead of just protein-protein interactions, since it has been shown that the CitA and PhoQ sensing domains both bind ligands. This discovery has lead to the hypothesis that other HKs may sense the environment using similar strategies.

1.5.2 The role of accessory proteins in regulation of two component signal transduction.

Some HKs sense a signal directly and elicit a cellular response. However, in many situations it appears that HKs use accessory proteins to help them respond appropriately to a given signal. These accessory factors are proteins that either have a positive affect on the pathway in response to a signal or negatively regulate the pathway.

In the case of positively acting accessory factors, the majority identified to date are lipoproteins. NlpE is one these positively acting lipoproteins. Otto and Silhavy (2002) showed that NlpE is needed for the Cpx pathway to sense adhesion to hydrophobic surfaces, because when it is absent the Cpx pathway no longer responds to this signal. However, NlpE is not needed by the pathway to sense other inducing cues like alkaline pH (DiGiuseppe & Silhavy, 2003), suggesting that NlpE senses and produces an adhesion specific signal that the Cpx pathway responds to (Otto & Silhavy, 2002). The exact mechanism for how this happens is not known, although no interaction between CpxA and NlpE has been shown. Another lipoprotein, RcsF, which regulates the Rcs two component pathway functions similarly to NlpE. It has been shown that RcsF is needed for the RcsC HK to sense specific conditions such as low temperature, the presence of acidic phospholipids, excess Zn^{2+}, and affects on lipopolysaccharides (Majdalani *et al.*, 2005). However, RcsF is not needed for RcsC to sense all activating cues of the Rcs pathway, such as overexpression of DjlA (Castanie-Cornet *et al.*, 2006). Just as the mechanism for how NlpE allows CpxA to sense adhesion is not known, the mechanism for how RcsF relays the message to RcsC about perturbations to the cell surface has also yet to be determined. It has been shown that both RcsF and NlpE can induce their respective pathways when overexpressed (Majdalani *et al.*, 2005, Snyder *et al.*, 1995). It was also shown that overexpression of two other lipoproteins, YpdI and LolA (Chen *et al.*, 2001, Potrykus & Wegrzyn, 2004) and an inner membrane bound protein with chaperone activity, DjlA (Clarke *et al.*, 1997, Kelley & Georgopoulos, 1997) induce the Rcs pathway through RcsC. Thus, these proteins may also be needed for the Rcs pathway to sense specific conditions that have yet to be discovered.

Lipoprotein accessory factors have also been identified and implicated in TCST systems in Gram positive bacteria. In *Bacillus subtilis*, KapB, a lipoprotein, is needed for the HK, KinB, to sense the trigger for sporulation (Dartois *et al.*, 1997). Unlike Gram negatives, where only positively regulating lipoproteins have been identified, negatively regulating lipoproteins have been found in Gram positives. In *Streptomyces coelicolor*, the CseBC system, which controls the expression of σ^E activity in response to cell wall stress (Hong *et al.*, 2002, Paget *et al.*, 1999), is regulated by a third component CseA (Hutchings *et al.*, 2006). CseA is a lipoprotein that negatively regulates the CseBC TCST system, since when CseA is deleted there is an increase in expression of the *sigE* promoter (Hutchings *et al.*, 2006). An interesting aspect of the CseBC and CseA system is that they are encoded in the same operon along with *sigE* (Paget *et al.*, 1999). There are five other TCST operons that are associated with lipoproteins in the *S.coelicolor* genome (Hutchings *et al.*, 2006). From what is known about NlpE, RcsF, KapB and CseA, one could hypothesize that these lipoproteins may help regulate activity of the TCST systems in the corresponding operon by inhibition or by activation under a specific condition. Either way these lipoproteins could be added to the fast growing list of lipoproteins that help TCST systems sense their surroundings.

Many proteins have been characterized as negative accessory factors of both TCST and other signal transduction systems. Probably the best known negative accessory protein is RseB, which belongs to the σ^E signal transduction pathway (De Las Penas *et al.*, 1997a, Missiakas *et al.*, 1997, Collinet *et al.*, 2000). RseB has been shown to interact with RseA, protecting RseA from proteolysis in the absence of a stress signal (De Las Penas *et al.*, 1997a, Missiakas *et al.*, 1997, Collinet *et al.*, 2000, Ades *et al.*, 1999, Cezairliyan & Sauer, 2007). Ultimately this increases the stability of the RseA-σ^E interaction, preventing activation of the pathway in the absence of stress (Collinet *et al.*, 2000, Grigorova *et al.*, 2004). Another protein that parallels RseB in function is TcpH in *Vibrio cholerae*. TcpH protects TcpP, an inner membrane transcription factor, from degradation (Beck *et al.*, 2004). It was shown that a mutant lacking TcpH had undetectable levels of TcpP (Beck *et al.*, 2004), which controls the expression of *toxT*, the transcriptional activator of cholera toxin and pilus production (DiRita & Mekalanos, 1991, Hase & Mekalanos, 1998),explaining why these strains are avirulent. Another negative accessory protein is CpxP of the Cpx pathway. CpxP downregulates the Cpx pathway 3 to 5 fold through a hypothesized interaction with the HK, CpxA (Raivio *et al.*, 2000, Raivio *et al.*, 1999), and when it is deleted there is a slight increase in pathway activity (Raivio *et al.*, 1999). Furthermore, the titration or depletion of CpxP is proposed to be key to the activation of the pathway, since in the presence of spheroplasts, an inducer of the Cpx response, IM tethered CpxP does not allow for the activation of the pathway (Raivio *et al.*, 2000).

The Rcs envelope stress pathway also contains an inhibitory protein, IgaA (Cano *et al.*, 2002). *igaA* is essential in *E.coli*, however an *igaA1* allele was isolated that contained a point mutation that caused a loss of function phenotype (Cano *et al.*, 2002). When cells contained this allele it was shown that Rcs pathway activity was increased (Cano *et al.*, 2002). A direct interaction between IgaA and RcsC, the HK, or RcsB, the RR, has not been shown, but genetic evidence does suggest a physical interaction because RcsBC$^-$ can suppress the lethality an IgaA$^-$ mutant (Cano *et al.*, 2002). It is thought that IgaA is needed to repress the Rcs pathway in order to prevent attenuation in virulence because it was shown in *Salmonella* that virulence was attenuated when the Rcs pathway was activated (Dominguez-Bernal *et al.*, 2004).

The lists of negatively acting accessory factors in TCST systems are ever growing and are emerging in all organisms as a way fine of tune signaling. The SenSR system in *E.coli*, which responds directly or indirectly to redox changes, is negatively regulated by HbpS (Ortiz de Orue Lucana *et al.*, 2005). It was shown that HbpS binds directly to SenS, the HK, to negatively regulate the system. The FixLJ system in *Sinorhizobium meliloti* which regulates nitrogen fixation and respiration in response to low oxygen levels, is negatively regulated by FixT (Garnerone *et al.*, 1999). The negatively regulating accessory proteins, YycI and YycH, have also been shown to inhibit the expression of the essential TCST system, YycGF in *B. subtilis* that is involved in cell wall metabolism and cell division

(Szurmant *et al.*, 2007a). A direct interaction between the transmembrane domains of YycI and YycH with the transmbmerane domain of YycG, the HK has been demonstrated (Szurmant *et al.*, 2007b). Recently a TCST system, ApsSR, was discovered in *Staphylococcus epidermidis* that responds to antimicrobial peptides (AMPs) (Li *et al.*, 2007). The ApsSR system contains a third component that is cytoplasmic, ApsX, that is indispensable for sensing AMPs (Li *et al.*, 2007). Why ApsX is needed to sense AMPs has yet to be determined but it is most likely that it acts as an accessory protein that helps the Aps system sense AMPs (Li *et al.*, 2007).

Thus, it now seems that many TCST systems can be expanded from the typical two components of a HK and RR to having three or four components that help in sensing and responding to a given signal. The addition of these accessory factors to TCST systems may help control the specificity of the response to avoid overlap between pathways or they may help to amplify the signal before it becomes detrimental to them. These accessory factors also provide multiple points for signal input and control so that TCST systems can respond to multiple signals.

1.5.3 How do response regulators regulate gene expression?

In response to environmental stimuli, bacteria activate or repress target genes to adapt. For TCST systems this activation/repression is carried out by the RR. The most orthodox RR contains a receiver domain and a DNA binding domain. The receiver domains of different RR share 20 to 30% homology, with the most conserved region being the acid pocket, which contains three conserved aspartates and a lysine residue (Hakenbeck & Stock, 1996, Stock *et al.*, 2000). This acid pocket is involved in binding and stabilizing the phosphate that is transferred from the HK to the cognate RR, and has been implicated in contributing to the conformation change that takes place upon phosphorylation of the RR (Stock *et al.*, 2000, Hakenbeck & Stock, 1996, Foussard *et al.*, 2001). Upon activation of the HK by phosphorylation the phosphate is transferred to one of the conserved aspartate residues in the acid pocket. This phosphate transfer is catalyzed by the RR itself (Foussard *et al.*, 2001). In some cases it is thought that when the receiver domain is unphosphorylated it interferes with the DNA binding domain, inhibiting it. Upon activation by phosphorylation, it is thought that there is a conformational change in the RR that relieves the inhibition between the DNA binding domain and the receiver domain (Gu *et al.*, 1994). In other cases it is thought that the phosphorylation of the RR promotes activation by allowing dimerization, higher order oligomerization, or protein-protein interaction (Stock *et al.*, 2000). In any case the phosphorylation of the RR is thought to shift it into an active conformation.

Once the RR is activated by phosphorylation, the dimerized RR binds to DNA to activate or repress gene expression (Hakenbeck & Stock, 1996, Foussard *et al.*, 2001). Activation of the regulon can occur through an interaction with the α-subunit of the RNAP, or via σ factors where the RR acts as a transcriptional enhancer (Stock *et al.*, 2000, Foussard *et al.*, 2001, Hakenbeck & Stock, 1996). Repression occurs when the RRs binding site occludes binding of RNAP or prevents the forward motion of the RNAP along the DNA (Stock *et al.*, 2000). Repression or activation of gene expression helps the cell adapt to a stimulus. Once the stimulus has been removed or dealt with the RR is dephosphorylated. The dephosphorylation of the RR can happen through three different mechanisms. Probably the most common is by its cognate HK in the phosphatase confirmation (Igo *et al.*, 1989). Dephosphorylation can also occur through accessory phosphatases or by the RR itself (Stock *et al.*, 2000). RRs that contain a DNA binding domain only make up 60% of known RRs. There are other types of output domains such as those involved in RNA, protein-binding, or ligand-binding. The output domains can also be involved in enzymatic activities. There are a few RRs that don't have an output domain and only contain a receiver domain (Galperin, 2006).

1.6 The Cpx response.

1.6.1 Discovery of the Cpx pathway and its components.

The Cpx (conjugative plasmid expression) pathway was first discovered in 1980 by McEwen and Silverman. They identified two genes, *cpxA* and *cpxB*, that when mutated caused an increased resistance to specific bacteriophage and an inability to synthesize F-pili (McEwen & Silverman, 1980a). It was also noted that *cpxA* mutants had altered envelope composition, with OmpF and murein lipoprotein either being absent or deficient (Weber & Silverman, 1988). Upon further investigation *cpxA* was mapped to minute 88 of the *E.coli* chromosome and shown to encode a 52 kDa protein that was located in the inner membrane with two hydrophobic regions and a periplasmic segment (McEwen & Silverman, 1980b, Weber & Silverman, 1988). Weber and Silverman (1988) proposed that CpxA was a transmembrane sensory protein. They also noted at this time that CpxA was the 3' gene of an operon (Weber & Silverman, 1988). Since it is known that most sensory proteins are found in an operon with their regulatory partner, Dong *et al.* (1993)

looked at the 2.1 kb segment upstream of *cpxA* and saw that it showed high homology to other response regulators and designated it CpxR. If CpxA and CpxR make up a cognate HK and RR pair, then one would expect a phosphotransfer event between them. Raivio and Silhavy (1997) showed that CpxA has both kinase and phosphatase activities for CpxR. DeWulf and Lin (De Wulf & Lin, 2000) showed in *E.coli* that deletion of *cpxR* relieved all the phenotypes of a constiutively active *cpxA** allele, suggesting that CpxA is not able to cross-talk with any additional RRs and that CpxR is its only cognate RR. However it was suggested in *Salmonella* that CpxA may be responsible for cross-signaling additional RRs (Nakayama *et al.*, 2003). Nakayama *et al.* (2003) suggested that CpxA signaled/communicated with two different RRs based on the observation that in a *cpxA* null background there was a decrease in *hilA* expression, but in a *cpxR* null background the expression pattern of *hilA* was similar to that of wild type. One could argue that this expression pattern of *hilA* is not do to a cross-signaling event but is a characteristic regulation pattern of two component systems. It is known that in a *cpxA* null mutant, CpxR cannot be dephosphorylated but can still be phosphorylated by small phosphate donors (Danese *et al.*, 1995). So what might be happening with *hilA* is that CpxR~P represses *hilA* expression, which is what was observed in a *cpxA* null strain. Conversely in *cpxR* null strain, wild type *hilA* transcription occurs with no repression from the Cpx pathway. This would suggest that CpxA is not responsible for cross-signaling other RRs and that CpxR is the only cognate RR of CpxA.

It was also discovered that the Cpx pathway is not a typical two component system, because of a third component, the periplasmic inhibitor protein, CpxP (Danese & Silhavy, 1998, Raivio *et al.*, 1999). CpxP is secreted to the periplasm via the Sec system, thus the first 21 amino acids of CpxP contain a typical signal sequence, which contains an abasic region, hydrophobic middle section and a C-terminal cleavage site (Figure 1-2). Once secreted into the periplasm the mature form of CpxP is 145 amino acids in length, with a molecular weight of approximately 18.9 kDa and a pI of 7.48. The data to date suggest that the Cpx pathway is a modified TCST system that monitors the envelope using CpxA and CpxP, and that the cellular response is propagated through CpxR.

1.6.2 Activating cues of the Cpx pathway.

Understanding what the Cpx pathway responds to and monitors will ultimately allow one to determine its role within the cell. From the discovery of CpxA, it was determined that CpxA mutants exhibited alterations to the envelope. Initially, it was shown in *Shigella* that pH affected the expression of *virF* in a *cpxA* dependent manner (Nakayama & Watanabe, 1995). They showed that at a pH of 6.0 *virF* expression was repressed but at a pH of 7.4 it is activated, suggesting that pH affects the Cpx pathway. Danese and Silhavy (1998) went on to show that the Cpx pathway was activated by alkaline pH in a *cpxA* dependent manner. Exactly what pH is doing to the cell to activate the Cpx response is not understood. It is thought that it may be causing misfolded proteins within the envelope, but ultimately pH could be causing numerous stresses that the Cpx pathway is responding to.

Snyder *et al.* (1995) overexpressed a tripartite periplasmic fusion protein, LamB-LacZ-PhoA, that exerts a toxicity of unknown origin on the envelope. They found that they could suppress the toxicity of LamB-LacZ-PhoA by overproducing NlpE (Snyder *et al.*, 1995), which in turn activates the Cpx response. It is suggested that overproducing NlpE causes mislocalized or misfolded NlpE, which is recognized by CpxA, causing upregulation of the Cpx regulon, which alleviates the toxicity of LamB-LacZ-PhoA (Snyder *et al.*, 1995). It was also shown that the Cpx pathway senses and responds to overexpression of P-pili subunits, PapG and PapE, that are misfolded because of the absence of their periplasmic chaperone, PapD (Jones *et al.*, 1997). Not only does the Cpx pathway respond to P-pili subunits but it also responds to the overexpression of the enteropathogenic *E.coli* type IV bundle forming pilus subunit, BfpA (Nevesinjac & Raivio, 2005). It is known that P-pili subunits that are driven off pathway aggregate on the periplasmic face of the IM, that pre-BfpA subunits are found in pools within the IM, and the overexpression of NlpE causes the protein to mislocalize to the periplasmic face of the IM. All these observations suggest that the Cpx pathway is responding to misfolded proteins in the envelope, specifically those that are associated with the periplasmic face of the IM (Figure 1-3). It was also determined that alterations to the envelope, such as a lack of phosphatidylethanolamine or the accumulation of enterobacterial common antigen intermediate lipid II, induced the Cpx pathway (Mileykovskaya & Dowhan, 1997, Danese *et al.*, 1998). As well, removing the OM and periplasm by spheroplasting also leads to the induction of the pathway (Raivio *et al.*, 2000). Thus general perturbations to the envelope also induce the Cpx response.

The Cpx response is also induced upon bacterial contact with hydrophobic abiotic surfaces. This induction of the Cpx response is dependent on NlpE because in a *nlpE* deletion strain there are significantly lower numbers of cells attached to the surface (Otto & Silhavy, 2002) and the Cpx response is not induced. The immediate activation of the Cpx pathway in response to attachment may be a check for the bacteria to combat or prevent envelope damage that may be caused by interactions between the bacteria and hydrophobic surfaces (Otto & Silhavy, 2002).

1.6.3 Inhibition of the Cpx pathway.

Raivio and Silhavy (1997) originally hypothesized that the Cpx pathway might be negatively regulated, since the removal of, or mutations to, CpxA's sensing domain caused constitutive activation of the pathway. It was thought that the sensing domain mutations caused CpxA to be "locked" into the activated state, unable to interact with the negative effector molecule under normal conditions. It was proposed that an interaction occured between the sensing domain of CpxA and a negative effector molecule (Raivio & Silhavy, 1997). Raivio *et al.* (1999) showed that this negative effector molecule was the Cpx regulated periplasmic protein CpxP. *cpxP* was first identified as a pH-regulated locus which encodes a periplasmic protein that helps overcome extracytoplasmic protein-mediated toxicity (Danese & Silhavy, 1998). Danese and Silhavy (1998) identified *cpxP* as a *lacZ* operon fusion that was up-regulated by NlpE in a CpxA-dependent manner. It was later shown that when CpxP is overexpressed it down regulates the pathway three to five fold (Raivio *et al.*, 2000, Raivio *et al.*, 1999). Further, inhibition was generated within the periplasm and required an intact CpxA sensing domain, since mutations or deletions in the sensing domain rendered CpxA signal blind to CpxP (Raivio *et al.*, 1999). Further, an inner membrane tethered MBP-CpxP protein can maintain Cpx pathway inhibition in the presence of spheroplasting, while a MBP-CpxP fusion localized to the periplasm does not (Raivio *et al.*, 2000). This data, along with the signal blind CpxA mutants, suggested that CpxP exerts its inhibitory effect through a direct interaction with the sensing domain of CpxA.

Recently, the Cpx pathway was reconstituted *in vitro* and it was shown that the addition of CpxP to proteoliposomes containing only CpxA was sufficient to inhibit the pathway (Fleischer *et al.*, 2007). Although Fleischer *et al.* (2007) did not show a direct protein-protein interaction this study excluded the involvement of any other proteins in the inhibition of the pathway and strongly suggested that there was a direct interaction between CpxP and CpxA. The inhibitory affect of CpxP on CpxA could be manifest in numerous ways. It could cause an to increase in CpxA phosphatase activity, removing the phosphate from CpxR more efficiently, it could affect the autophosphorylation of CpxA itself, or it could affect the phosphotransfer event between CpxA and CpxR, or any combination of these three (Raivio *et al.*, 1999). Fleischer *et al.* (2007) showed that when CpxP was present with CpxA in proteoliposomes, CpxA had a decreased ability to autophosphorylate itself but there was no affect on the phosphatase activity or phosphotransfer event. Currently it is thought that in the absence of envelope stress, CpxP interacts with the sensing domain of CpxA, inhibiting CpxA from autophosphorylating itself and maintaining the pathway in an off state (Figure 1-3). Upon activation, CpxP inhibition would be relieved, allowing for the rapid induction of the pathway (Figure 1-3). It has been shown that CpxP is not required for signal transduction since either in the absence of *cpxP* or in the presence of overexpression of CpxP, the Cpx pathway can still be further induced (Raivio *et al.*, 1999, DiGiuseppe & Silhavy, 2003). Thus the hypothesized role for CpxP is in fine-tuning the response, and it may be important in turn-off of the pathway.

1.6.4 The Cpx regulon helps alleviate envelope stress.

Upon activation, CpxA, like most HKs, autophosphorylates itself on a conserved histidine residue (Raivio & Silhavy, 1997, Fleischer *et al.*, 2007). Then there is a phosphotransfer event that is initiated by CpxR, and CpxR becomes phosphorylated, presumably on a conserved aspartate residue (Raivio & Silhavy, 1997, Fleischer *et al.*, 2007). The phosphorylated CpxR now has enhanced ability to bind to its consensus sequence and increase transcription of the Cpx regulon (Raivio & Silhavy, 1997, De Wulf *et al.*, 2002)(Figure 1-3).

The Cpx regulon includes numerous different protein folding factors, such as *dsbA* (major periplasmic disulfide oxidase), *ppiA* (peptidyl-prolyl-isomerase) and *degP* (Pogliano *et al.*, 1997, Danese & Silhavy, 1997, Cosma *et al.*, 1995, Danese *et al.*, 1995). This reaffirms the hypothesis that the Cpx pathway is involved in monitoring and regulating protein turn over and folding in the envelope. Along with the transcription of protein folding and degrading factors, the Cpx regulon also regulates expression of genes whose roles in responding to envelope stress are not understood (De Wulf *et al.*, 1999, De Wulf *et al.*, 2002). The Cpx pathway also amplifies the signal transduction genes *cpxRA* and *cpxP* in a positive autofeedback mechanism (Raivio *et al.*, 1999, De Wulf *et al.*, 2002). This autoamplification of *cpxRA* allows the commitment of the pathway to alleviate the stress at hand and the amplification of *cpxP* may help with the inhibition of the pathway once the stress has been relieved (discussed in a later section).

1.6.4.1 The Role of DegP in alleviating envelope stress.

The first gene identified as part of the Cpx regulon was *degP*. Danese *et al.* (1995) showed that *cpxA** strains caused an increase in *degP'-lacZ*$^+$ expression. It was also noted that the upregulation of *degP* in these *cpxA** strains helped relieve the toxicity caused when the toxic LamBA$_{23}$D and LamB-LacZ-PhoA proteins were overexpressed (Cosma *et al.*, 1995).

DegP was first identified by Strauch and Beckwith (1988) in a screen looking for mutants that stabilized a membrane bound hybrid protein that consisted of Tsr (chemosensory transducer) and AP (alkaline phosphatase). They showed that a *degP* mutant was also defective in clearing five other abnormal proteins found in the periplasm, but did not affect abnormal cytoplasmic proteins (Strauch & Beckwith, 1988). Further characterization of this new periplasmic protease showed that it was a serine endoprotease because of the limited number of cleavage fragments and the observation that serine protease inhibitors inhibited DegP but other inhibitors did not (Lipinska *et al.*, 1990). An interesting characteristic of DegP is that at a temperature of 30°C it takes on a chaperone activity but as the temperature increases from 30°C upwards the proteolytic activity rapidly increases in a non-linear fashion (Spiess *et al.*, 1999, Skorko-Glonek *et al.*, 1995). It is thought that at low temperatures DegP uses its chaperone activity to clear the accumulation of misfolded proteins by refolding, however at high temperatures DegP takes on a protease activity to clear misfolded proteins that accumulate and are unable to be refolded (Spiess *et al.*, 1999). The exact mechanism for how DegP cleaves its substrates and what determines the cleavage site within a substrate is not highly understood. It was determined that DegP cleaves at V/X or I/X sites, suggesting that DegP has a preference for small hydrophobic side chains at the P1 position (Kolmar *et al.*, 1996). However a hydrophobic side chain at the P1 position is not the only factor in determining where DegP cleaves. It was observed that PapA, a P-pilus subunit, was cleaved between two hydrophobic residues, confirming the cleavage site for DegP, however there are additional V/X and I/X sites within PapA that were not cleaved, suggesting additional determinants

(Jones *et al.*, 2002). DegP substrates must be either partially or completely unfolded prior to degradation because the hydrophobic residues that DegP recognizes are usually buried in the core of the protein (Kim *et al.*, 1999). Within the cell, DegP is found in a dodecamer form, with 2 hexamers stacked on top of each other, ultimately forming a central chamber where proteolysis is predicted to occur (Kim *et al.*, 1999, Krojer *et al.*, 2002). Each monomer of DegP contains two PDZ domains, designated PDZ1 and PDZ2, as well as the protease domain (Krojer *et al.*, 2002). It is thought that in the dodecamer form these PDZ domains are the gatekeepers, preventing axial access to the central cavity of DegP (Krojer *et al.*, 2002). It is thought that the hydrophobic residues of the substrate are bound by PDZ1 which contains a deep binding cleft for substrates with a pocket mainly made of hydrophobic residues. The substrate is then thought to be transferred to the inner cavity of DegP through lateral access, where it is degraded (Krojer *et al.*, 2002). However, recently it has been shown that the inner cavity of DegP is not essential for either the molecular chaperone or proteolytic activity (Jomaa *et al.*, 2007). Jomaa *et al.* (2007) showed that in a collapsed or reduced cage mutant, where the inner chamber is significantly smaller than 15Å, so that threading of the substrate into the inner chamber would be greatly compromised, DegP still showed significant protease activity. Thus it is now hypothesized that substrates access the proteolytic sites through a transient disassembly of the hexameric/dodecameric cage (Jomaa *et al.*, 2007). The reasoning for the stable hexameric and dodecameric form within the cell is to provide a safety mechanism, preventing uncontrolled proteolysis which would be hazardous to the cell (Jomaa *et al.*, 2007).

1.6.5 The role of the Cpx response.

Cells lacking the Cpx pathway showed no obvious phenotypes suggesting that this pathway may contribute to envelope homeostasis under a specific set of conditions (Connolly *et al.*, 1997), such as pH. The Cpx pathway is not only activated by pH, but in the absence of *cpxP* or *cpxR* the bacteria are sensitive to alkaline pH (Danese & Silhavy, 1998). This suggests that although the major role of the Cpx pathway has been linked to dealing with envelope stress due to misfolded and mislocalized proteins, over the years the Cpx response has been connected to pathogenesis in many bacteria such as *Shigella, Salmonella, Yersinia, Legionella*, enteropathogenic and uropathogenic *E.coli* (please refer to a review by (Raivio, 2005)). The Cpx pathway has been shown to be important in the early steps of infection, more specifically with adherence. As mentioned before the Cpx regulon includes numerous protein folding and degrading factors, more specifically DsbA and DegP, that play important roles in post-translational affects on pili (Donnenberg *et al.*, 1997, Jones *et al.*, 1997). Not only does the Cpx pathway regulate proteins needed for the proper assembly of virulence determinants but it is also important in the proper expression of virulence genes. In *Shigella*, it was shown that the Cpx pathway controls expression of the major virulence regulator (Nakayama & Watanabe, 1995, Nakayama & Watanabe, 1998, Mitobe *et al.*, 2005). Nakayama *et al.* (2003) also showed that CpxA was needed for the proper expression of *hilA*, which is a regulator of invasion in *Salmonella*. This may be why *Salmonella cpxA*$^-$ and *cpxA** mutants are significantly impaired in their ability to grow in mice (Humphreys *et al.*, 2004). As one can see there is a clear role for the Cpx pathway in pathogenesis, however its role does not stop there.

Other studies suggest the involvement of the Cpx pathway in processes like conjugation, adhesion to abiotic surfaces, motility/taxis, cell division, bioflim formation, and recovery and adaptation from/in stationary phase (Pogliano *et al.*, 1998, Dorel *et al.*, 1999, De Wulf *et al.*, 2002, De Wulf *et al.*, 1999, Otto & Silhavy, 2002, Jubelin *et al.*, 2005, Gubbins *et al.*, 2002, McEwen & Silverman, 1980a). Originally, the Cpx pathway was discovered because constitutively active CpxA mutants were shown to be defective in conjugation (McEwen & Silverman, 1980a). It was found that TraJ, the major regulator of conjugation, was unstable in a *cpxA** background, suggesting a mechanism by which the Cpx pathway prevents piliation and F-transfer during times of stress (Gubbins *et al.*, 2002). The Cpx pathway also inhibits the energy consuming processes of cell division, bioflim formation, and motility/taxis during times of stress. It was seen that *cpxA** mutants randomize the location of the FtsZ ring (Pogliano *et al.*, 1998). In addition, the Cpx pathway negatively regulates transcription of the *motABcheW* and *tsr*, genes involved in motility and chemotaxis, and Cpx$^-$ strains showed an increase in swarming rate (De Wulf *et al.*, 1999). Similarly when CpxR is activated there is a reduction in biofilm formation (Jubelin *et al.*, 2005). All of these studies support the idea that the Cpx response influences multiple cellular processes, although it is not always clear how the Cpx response exerts an effect on a given function.

1.7 Proposed research project.

Currently, a lot is known about the phosphotransfer reactions that happen between CpxA and CpxR and the nature of the Cpx regulon, but little is known about the way in which CpxA is activated or the role CpxP plays in response to envelope stress. CpxP is not required for signaling but is hypothesized to inhibit the Cpx pathway through an interaction with the sensing domain of CpxA, and possibly play a role in fine-tuning or shutting off the pathway (Figure 1-3). How CpxP inhibition is relieved, what its physiological role is in the cell, and how it interacts with CpxA are not understood. Understanding the role CpxP plays in Cpx signalling will ultimately allow the scientific world to better understand how the Cpx pathway senses and responds to envelope stresses and also how histidine kinases in general interact with accessory factors. It is therefore the objective of my project to take an in-depth look into the activation and repression of the Cpx pathway, with special attention to CpxP.

1.7.1 Characterization of the periplasmic inhibitor protein, CpxP.

1.7.1.1 Identification of functional domains in CpxP that are important for inhibition.

Since CpxP has no informative homologues it is unclear how CpxP exerts its inhibitory affects on the Cpx pathway. CpxP may be exerting its effects on the pathway in one of two ways; it may affect the autokinase/phosphatase activities of CpxA or it may be that overexpressing CpxP helps eliminate envelope stress. Either way the ultimate response is the down-regulation of the pathway. If CpxP is down-regulating the pathway because it is helping to eliminate envelope stress, one would think that overexpressing other members of the Cpx regulon that are involved in protein folding and degrading should also result in the down-regulation of the pathway. To test this I overexpressed, DsbA, PpiA, DegP, Spy (a periplasmic protein of unknown function) and CpxP. I then measured pathway activity using a *cpxP'-lacZ*$^+$ promoter fusion that is inserted in the chromosome at the λRS88 site, so as not to disrupt the native *cpxP* (Danese & Silhavy, 1998).

Whether CpxP is inhibiting the pathway through CpxA or by helping alleviate envelope stress, determining functional domains within CpxP will help us better understand how CpxP is down regulating the pathway. To identify functional domains within CpxP that are important for pathway inhibition, I used random mutagenesis of a CpxP-β-lactamase (CpxP'-'Bla) translational fusion construct encoded on pPB. I passaged pPB through an *E.coli* mutator strain called XL-1 Red (Stratagene) in hopes of introducing point mutations in *cpxP* so it is no longer able to inhibit the pathway. The mutated plasmids were then transformed into a strain carrying the *cpxP'-lacZ*$^+$ promoter fusion and screened for an increase in pathway activity due to a loss of inhibition by CpxP'-'Bla. To ensure that the point mutations did not cause missense mutations, colonies were screened to ensure that they maintained their Amp^{R} phenotype. Any potential loss-of-function mutants were then further characterized.

1.7.1.2 Determining if CpxP and CpxA interact.

It has been hypothesized in the literature that the way that CpxP inhibits the Cpx pathway is through a direct interaction with the sensing domain of CpxA. There are numerous pieces of genetic information that support this hypothesis. As mentioned before it has been seen that signal blind CpxA mutants can no longer be inhibited by CpxP, MBP-CpxP fusions tethered to the inner membrane can maintain the pathway in an off state even after spheroplasting, and the reconstituted Cpx pathway requires only CpxP and CpxA be present for down-regulation of the pathway (Fleischer *et al.*, 2007, Raivio *et al.*, 2000, Raivio *et al.*, 1999). In order to determine if CpxP and CpxA interact I employed numerous genetic and biochemical techniques, such as bacterial two-hybrid systems (Galperin, 2006), and cross-linking.

1.7.1.3 Dimerization of CpxP.

It is known that both HKs and RRs dimerize within the cell when activated (Stock *et al.*, 2000). It is then logical to think that accessory proteins that potentially interact with these HKs also form dimers within the cell. In collaboration with David C. Arthur and Ross A. Edwards from Dr. J.N. Mark Glovers' lab, we examined CpxP dimer formation *in vitro* and *in vivo*. For the *in vivo* experiments, we used cross-linking experiments to analyze if CpxP forms dimers *in vivo*. For *in vitro* experiments we used multi-angle laser light scattering, and small-angle X-ray scattering (SAXS) to look at the ability of CpxP to dimerize under repressive (pH 5.8) and activated (pH 8.0) conditions. We examined secondary and tertiary structure changes at these pHs using Far-UV circular dichroism (CD) and Near-UV CD, respectively.

1.7.1.4 Determining a physiologically relevant role for CpxP.

Throughout the literature (Raivio *et al.*, 1999, Raivio *et al.*, 2000), the function of CpxP has been characterized by overexpression, either in native form or as a fusion protein. This has lead to an in depth characterization of CpxP but has not addressed the physiological role of CpxP. I developed an assay that looks at the function of CpxP and does not depend on overexpression. The goal of this assay was to examine wild type and *cpxP* null strains for differences in activation and inhibition of the pathway under various inducing conditions, such as high pH and during overexpression of NlpE. I followed Cpx pathway activity using different Cpx promoters fused to the luciferase genes of *Vibrio* spp. I focused on the initial activation and repression of the pathway, as I would hypothesized that if CpxP is important in fine tuning the response, one would see the affects of CpxP during early activation or repression.

1.7.2 Screen for additional regulators of the Cpx pathway.

In Gram negative bacteria stress responses have been split into the cytoplasmic and extracytoplasmic (envelope) responses. This suggests that these responses are completely separate and that there is no cross regulation between the two of them. Recently, it was shown that ppGpp is needed for the activation of the σ^E pathway in stationary phase and this activation is independent of RseA (Costanzo & Ades, 2006), suggesting that there is communication between the cytoplasmic and extracytoplasmic stress responses and that they are not completely separate responses. It has also been known for a long time that overlap exists between the envelope stress responses, either with the inducing cues that they sense or in the genes that they regulate. With respect to the Cpx pathway it has been shown that it co-regulates *degP* and *spy* with the σ^E and Bae pathways, respectively (Raffa & Raivio, 2002, Danese & Silhavy, 1997). As well the Cpx response is induced by PapG, which also induces the σ^E and the Bae pathways (Jones *et al.*, 1997, Raffa & Raivio, 2002). These observations suggest that there is some level of cross communication between different regulatory systems. This cross communication may help the bacteria adapt to changing environments faster or prepare the bacteria for further stresses, which will ultimately result in an increased survival rate. With respect to σ^E and ppGpp, it appears that σ^E is able to respond to both internal and external environments. Our lab, as well as others (DiGiuseppe & Silhavy, 2003), have noticed a growth dependant activation of the Cpx pathway upon entry into stationary phase that is independent of CpxA, suggesting that the Cpx pathway may be cross regulated like σ^E. To identify potential regulators of the Cpx pathway, I used a transposon mutagenesis screen. I looked for transposon mutants that caused a decrease in *cpxP'-lacZ*$^+$ activity in stationary phase. Interesting mutants were identified via sequencing and BLAST searches. They were further characterized by determining their epistasis with other Cpx pathway regulators and I attempted to elucidate a possible mechanism for their effects on *cpxP'-lacZ*$^+$ expression.

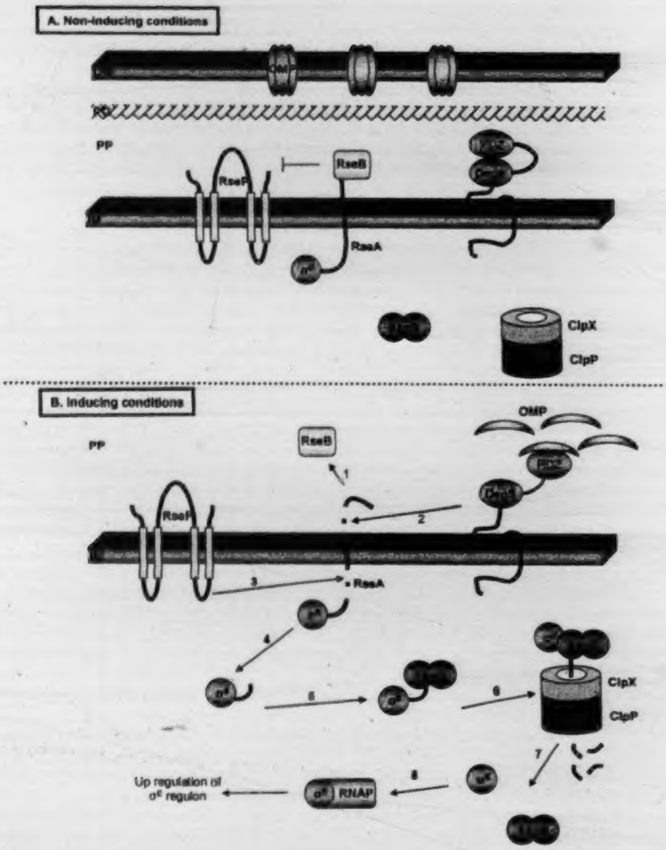
A. Non-inducing conditions
PP
RseP
RseB
RseA
σ^E
ClpX
ClpP
B. Inducing conditions
OMP
PP
RseB
1
RseP
2
3
RseA
4
5
6
ClpX
ClpP
7
8
σ^E
σ^E RNAP
Up regulation of σ^E regulon

Figure 1-1. Regulated intramembrane proteolysis of the σ^E pathway (adapted from MacRitichie *et al.*, 2007). A) Under non-inducing conditions RseB stabilizes the interaction between RseA and σ^E by binding to the periplasmic side of RseA, inhibiting the proteolysis of RseA by DegS and RseP. Also to insure that there is no premature degradation of RseA by DegS, the PDZ domain of DegS inhibits its protease domain. B) Under inducing conditions, RseB is titrated away by a mechanism that is still unknown (1). The YFF peptide in misfolded OMPs interacts with the PDZ domain of DegS, relieving the inhibition the PDZ domain exerts on the protease domain. DegS then cleaves RseA on the periplasmic side of the transmembrane domain (2). This truncated form of RseA then acts as a substrate for cleavage by RseP (3). The cytoplasmic domain of RseA that is still bound by σ^E is now released in the cytoplasm (4), where SspB binds it (5) and delivers it to ClpXP (6). ClpXP degrades the cytoplasmic domain of RseA, releasing σ^E (7). σ^E is now free to bind with RNAP (8) and upregulate the σ^E regulon. The numbered arrows indicate the order of how σ^E is activated. OM, outer membrane, IM, inner membrane, PP, periplasm, PG, peptidoglycan, OMP, outer membrane proteins, RNAP, RNA polymerase.

```
MRIVTAAVMASTLAVSSLSHAAEVGSGDNWHPGEELTQRSTQSHMFDGISLTEHQRQQ    58
•••••••••••••••••••••••

MRDLMQQARHEQPPVNVSELETMHRLVTAENFDENAVRAQAEKMANEQIARQVEMAKV   116

RNQMYRLLTPEQQAVLNEKHQQRMEQLRDVTQWQKSSSLKLLSSSNSRS            165
```

Figure 1-2. The full length sequence of CpxP. The first 21 amino acids (underlined in black) represent the Sec-dependent signal sequence.

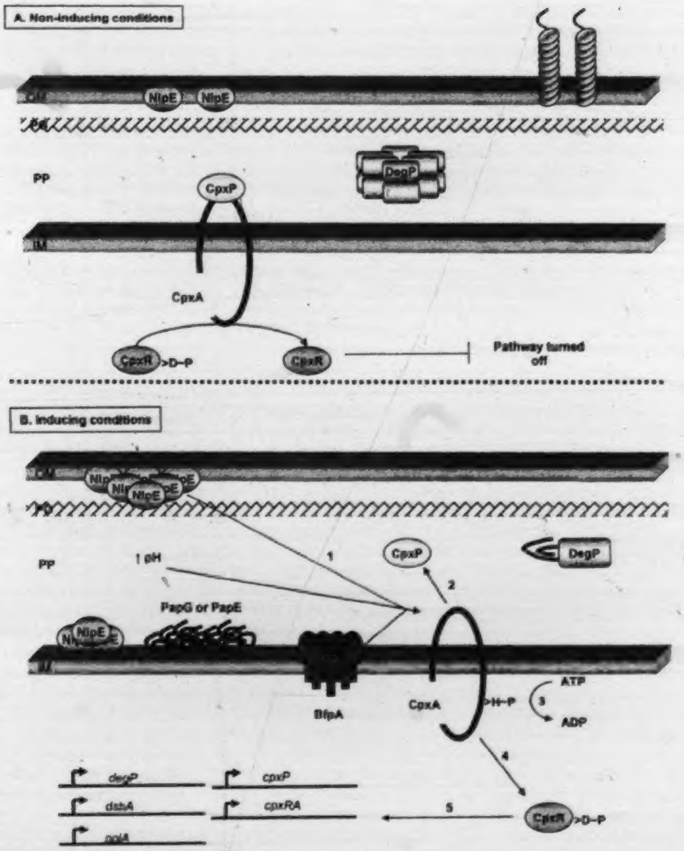
A. Non-inducing conditions
OM
NlpE
NlpE
PG
PP
CpxP
DegP
IM
CpxA
CpxR >D–P
CpxR
Pathway turned off
B. Inducing conditions
OM
NlpE
PG
PP
↑ pH
1
CpxP
DegP
2
PapG or PapE
NlpE
IM
BfpA
CpxA
>H–P
ATP
3
ADP
4
degP
cpxP
dsbA
cpxRA
5
CpxR >D–P
ppiA

Figure 1-3. Regulation of the Cpx pathway. A) Under non-inducing conditions it is hypothesized that CpxP interacts with the sensing domain of CpxA, inhibiting the autokinase activity of CpxA. During this time CpxA uses the phosphatase activity to remove any phosphates on CpxR, maintaining the pathway in an off state. B) Under inducing conditions, CpxA senses stresses via the periplasmic sensing domain (1), CpxP is then titrated either alone or in conjunction with misfolded proteins freeing the sensing domain of CpxA to sense the stresses present (2). CpxA then autophosphorylates itself on a conserved H using ATP (3). The phosphate is then transferred to CpxR, via a phosphtransfer event that is initiated by CpxR (4). The phosphorylated form of CpxR then goes on to regulate the Cpx regulon to help deal with and eliminate the stressors (5). The numbered arrows indicate the order in which the Cpx pathway is activated. IM, inner membrane, OM, outer membrane, PG, peptidoglycan, PP, periplasm, D, aspartic acid, H, histidine, ATP, adenosine triphosphate, ADP, adenosine diphosphate, P, phosphate.

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Chapter 2:

Cpx signal transduction is influenced by a conserved N-terminal domain in the novel inhibitor CpxP and the periplasmic protease DegP*

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2.1 Introduction.

Constantly changing environments are a critical situation to which all bacteria must adapt in order to survive. In Gram negative bacteria the envelope, consisting of the outer membrane, periplasm and inner membrane, is in constant contact with the environment. *Escherichia coli* has at least three regulatory pathways, the σ^E stress response, the CpxRA and the BaeSR two-component systems, which are activated by, and mediate adaptation to, different envelope stresses (Ades, 2004, Alba & Gross, 2004, Raffa & Raivio, 2002, Raivio & Silhavy, 1999). The Cpx signal transduction pathway is a typical two-component system with a membrane bound histidine kinase, CpxA, and a cytoplasmic response regulator, CpxR (Dong *et al.*, 1993, Weber & Silverman, 1988). The activating cues of the Cpx pathway include alterations in extracellular pH (Danese & Silhavy, 1998, Nakayama & Watanabe, 1995), accumulation of enterobacterial common antigen (ECA) intermediate lipid II (Danese *et al.*, 1998), overexpression of NlpE (Snyder *et al.*, 1995), overexpression of P pilus subunits in the absence of their periplasmic chaperone, PapD (Jones *et al.*, 1997) or overexpression of the enteropathogenic *E.coli* (EPEC) type IV bundle forming pilus (BFP) subunit, BfpA (Nevesinjac & Raivio, 2005) . Each of these activating cues are expected to lead to the accumulation of misfolded and/or mislocalized proteins associated with the envelope, which are likely a component of the activating signal for the Cpx pathway. When activated, CpxA acts as a histidine autokinase (Raivio & Silhavy, 1997). The phosphorylated CpxA then transfers the phosphate to a conserved aspartate on CpxR (Raivio & Silhavy, 1997). Phosphorylated CpxR has enhanced ability to bind to consensus sequences and increase transcription of the Cpx regulon (Danese & Silhavy, 1997, Pogliano *et al.*, 1997, Raivio & Silhavy, 1997), which contains numerous envelope protein folding and degrading factors, and a variety of other genes whose roles in responding to envelope stress are not understood (De Wulf *et al.*, 2002, Raivio & Silhavy, 1999). Among the envelope protein folding and degrading factors induced are the periplasmic endoprotease, DegP (Danese *et al.*, 1995, Lipinska *et al.*, 1990), two peptidyl-prolyl-isomerases, PpiA and PpiD (Dartigalongue & Raina, 1998, Liu & Walsh, 1990, Pogliano *et al.*, 1997), and DsbA, the major periplasmic disulfide oxidase (Bardwell *et al.*, 1993, Danese & Silhavy, 1997, Kamitani *et al.*, 1992, Pogliano *et al.*, 1997). Along with increased transcription of the protein folding and degrading factors, a small periplasmic inhibitor protein, CpxP, is also expressed at elevated levels, together with the *cpxRA* genes (Danese & Silhavy, 1998, Raivio *et al.*, 2000, Raivio *et al.*, 1999). Thus, a major role of the Cpx response appears to be in maintaining envelope proteins under adverse conditions.

cpxP was first identified as a pH-regulated locus which encodes a periplasmic protein that helps overcome extracytoplasmic protein-mediated toxicity (Danese & Silhavy, 1998). Danese and Silhavy (1998) identified *cpxP* as a *lacZ* operon fusion that was up-regulated by NlpE in a CpxA-dependent manner. Furthermore, CpxP is involved in signal transduction since overexpression of CpxP causes a three to five fold reduction in Cpx-mediated gene expression via the periplasmic sensing domain of CpxA (Raivio *et al.*, 2000, Raivio *et al.*, 1999). An inner membrane tethered MBP-CpxP protein can maintain Cpx inhibition in the presence of spheroplasting, a strong Cpx-activating signal, while a MBP-CpxP fusion localized to the periplasm does not, suggesting that the CpxA:CpxP interaction is direct (Raivio *et al.*, 2000). Currently it is thought that in the absence of envelope stress, CpxP interacts with the sensing domain of CpxA, maintaining the pathway in an off state. Upon activation, CpxP inhibition would be relieved, allowing for induction of the response. However, CpxP is not required for signal transduction since in either the absence, or presence of overexpression, of CpxP, the Cpx pathway can still be further induced (DiGiuseppe & Silhavy, 2003, Raivio *et al.*, 1999). Thus, the hypothesized role of CpxP is in fine-tuning the response.

In this study we address the question of how CpxP-mediated inhibition might occur and be relieved. Since CpxP has no informative homologues, we set out to identify possible functional domains in CpxP that are important for signal transduction. Using a translational CpxP'-'Bla fusion construct, we identified a highly conserved, predicted α-helix in the N-terminal domain of CpxP that affects both the inhibitory function and stability of the protein. Diminished levels of some of the loss-of-function mutants relative to the wild type CpxP'-`Bla protein suggested that proteolysis might affect CpxP-mediated inhibition. Indeed, we noted that the levels of the mutant CpxP'-'Bla proteins could be returned to, or elevated above, normal in the absence of DegP. DegP proteolysis is likely important for controlling CpxP levels in response to inducing cues since *degP* mutation simultaneously abrogates the disappearance of CpxP'-`Bla and diminishes pathway activation at elevated pH. We propose that the predicted N-terminal α-helix is important for the CpxA-dependent inhibition of the pathway and that CpxP levels are controlled by DegP-dependent proteolysis.

2.2 Material and Methods.

2.2.1 Bacterial strains and plasmids.

Stains and plasmids used in this study are listed in Table 2-1 and Table 2-3, respectively. All strains were constructed using standard genetic techniques (Silhavy *et al.*, 1984). PCR primers are described in Table 2-3.

2.2.2 Media, antibiotics and growth conditions.

All strains were grown on Luria-Bertani (LB) agar (Silhavy *et al.*, 1984) or lactose-MacConkey agar (Difco), which is an indicator media that permits measurement of β-galactosidase levels, at either 37° or 30°C. Liquid cultures were grown in LB broth with aeration. Strains were maintained with the appropriate selection, 100μg/mL ampicillin (Amp), 100μg/mL chloramphenicol (Cam), 25μg/mL kanamycin (Kn) or 25μg/mL tetracycline (Tet). All antibiotics were purchased from Sigma.

2.2.3 Mutagenesis of pPB.

Random *cpxP* point mutants were isolated by passaging pPB through DNA repair deficient strain XL-1 Red (Stratagene), according to the manufacturer's specifications. Twenty independently derived samples of mutagenized pPB were collected after passaging through XL-1 Red and were transformed into TR50. Transformants were grown at 37°C for a maximum of 20 hours on lactose-MacConkey media containing ampicillin. Lac$^+$, Amp^R derivatives were isolated and further characterized.

2.2.4 DNA sequence analysis.

Mutated pPB plasmid DNA was extracted using a QIAprep Spin Mini Prep Kit (Qiagen), according to the manufacturer's protocol. The mutated *cpxP* genes were amplified using the CpxP*Bla* and CpxP5'*Kpn* primers (Table 2-3). The PCR products were purified using a Qiagen PCR purification kit (Qiagen), according to the manufacturer's directions. Sequencing was carried out using the protocol for the DYEnamic ET terminator cycle sequencing premix kit (Amersham) provided by the Molecular Biology Service Unit (University of Alberta).

2.2.5 β-galactosidase assay.

Single colonies of each bacterial strain to be assayed were inoculated into 2 mL overnight cultures of LB broth containing the appropriate antibiotics at 37° or 30°C with aeration. The next day the cultures were diluted 1:50 into fresh medium and grown at 37° to late log phase (O.D.$_{600}$ = 0.6). Induction of the Cpx response by elevated pH was accomplished by adding 1M sodium phosphate buffer of the appropriate pH (Sambrook *et al.*, 1989) to the media to a final concentration of 100mM. β-galactosidase activity was measured using the microtiter plate assay (Slauch & Silhavy, 1991) with slight variation to the protocol; 5μL of cell mixture was added to 195μL of Z-buffer due to high expression of β-galatosidase in the strains. The final calculation was then multiplied by 10 to account for the dilution. Each assay was performed in triplicate. Error bars represent the standard deviation.

2.2.6 Western blot analysis.

Overnight cultures of the appropriate strains grown in LB plus antibiotics at 30°C or 37°C with aeration were subcultured 1:50 and grown at 37° for 4 hours. pH induction of the Cpx response was accomplished using the same procedure described for the β-galactosidase assays above. The O.D.$_{600}$'s of the cultures were measured and whole cell extracts were prepared by pelleting volumes that were adjusted to correspond to 1mL of the culture that reached the lowest O.D.$_{600}$. Cell extracts were resuspended in 50μL of 2X SDS-PAGE loading dye (Sambrook *et al.*, 1989). Lysates were boiled for 5 min and 10μL were electrophoresed on a 10% SDS-PAGE minigel system (BioRad) (Laemmli, 1970). Proteins were transferred to nitrocellulose membrane at 10V overnight (Towbin *et al.*, 1979). The membrane was labelled as previously described by Raivio *et al.* (1999). β-lactamase monoclonal antibody (1:1 000 dilution) (QED BioScience Inc.) or bacterial alkaline phosphatase polyclonal antibody (1:20 000 dilution) (Research Diagnostics, Inc.) were used as primary antibodies. A secondary anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) was used at a 1:10 000 dilution. Proteins were visualized using the Immun-Star^{TM} AP substrate pack chemiluminescent kit (BioRad).

2.2.7 Site-directed mutagenesis (SDM) of pCpxPU to make pCpxPD$_{61}$EU and pCpxPQ$_{55}$PU.

The D$_{61}$E and Q$_{55}$P mutations were introduced into the pCpxPU vector as follows. First, the genomic region surrounding *cpxP* was PCR amplified using the CpxPBam and CpxPKpn primers and cloned into the BamHI and KpnI sites of pUC18 to construct pCpxPU. Next, the D$_{61}$E and Q$_{55}$P mutations were introduced into pCpxPU using the primers D-61-E1 and D-61-E2 (Table 2-3) for pCpxPD$_{61}$EU and the primers Q-55-P1 and Q-55-P2 (Table 2-3) for pCpxPQ$_{55}$PU using the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with slight variations to the protocol as follows. 2μL of 10mM dNTPs was used in the extension reaction for 6.5 minutes at 68°, 1μL of *Dpn*I (5U/μL) was used to digest unmutated template DNA for 2.5 hours, One Shot Top10 chemical competent cells (Invitrogen) were used in place of the XL-1 Blue competent cells, 5 μL of *Dpn*I-treated DNA was added to the Top10 cells, and LB was used in replace of NZY$^+$ broth.

2.3 Results.

2.3.1 Inhibition of the Cpx pathway is specific to CpxP overexpression.

Raivio *et al.* (1999 and 2000) have shown that overproduction of CpxP represses the Cpx regulon in a CpxA-dependent manner. From these findings it was proposed that CpxP acts as a periplasmic inhibitor by interaction with the sensing domain of CpxA. Alternatively it is possible that CpxP overexpression may lead to pathway inhibition by aiding in envelope protein folding, thus relieving envelope stress and causing down-regulation of the Cpx response in this fashion (DiGiuseppe & Silhavy, 2003). If this is true, then overproduction of other Cpx-regulated members may also down-regulate the pathway.

To test this hypothesis, we overexpressed numerous members of the Cpx regulon: a periplasmic endoprotease, DegP (Kolmar *et al.*, 1996, Lipinska *et al.*, 1990), a major disulfide oxidase, DsbA (Bardwell *et al.*, 1993, Kamitani *et al.*, 1992), a peptidyl-prolyl-isomerase, PpiA (Liu & Walsh, 1990), a small periplasmic protein of unknown function, Spy (Hagenmaier *et al.*, 1997), and CpxP (Danese & Silhavy, 1998) from p*trc*99A (Pharmacia). Overexpression of CpxP, PpiA and Spy was confirmed by SDS-PAGE analysis of isopropylthiogalactopyranoside (IPTG)-induced periplasmic extracts (data not shown). DegP and DsbA overexpression was confirmed by complementation of temperature sensitivity (Strauch *et al.*, 1989) and DTT (DL-Dithiothreitol) sensitivity (Dartigalongue *et al.*, 2000), respectively, of *degP* and *dsbA* null mutants, expressing either pDegP or pDsbA (data not shown) .

To see what effect overexpression of these factors may have on Cpx-regulated gene expression we used a *cpxP'-lacZ*$^+$ fusion in single copy on the chromosome. When CpxP was overexpressed there was a five-fold reduction in Cpx-mediated gene expression, as expected (Figure 2-1, compare lanes 1 and 2). In contrast, overexpression of DegP, DsbA, PpiA or Spy had no effect on Cpx-mediated gene expression (Figure 2-1, compare lanes 1 with 3-6). We conclude that CpxP functions uniquely as an inhibitor of the Cpx response.

2.3.2 Isolation of *cpxP* mutants.

Since CpxP has no informative homologues, we sought to identify functional domains in CpxP that are important for signal transduction. The strongest phenotype available to monitor CpxP function is the inhibition of Cpx-regulated gene expression upon CpxP overexpression (DiGiuseppe & Silhavy, 2003, Raivio *et al.*, 2000, Raivio *et al.*, 1999). Thus, we looked for *cpxP* loss-of-function mutations that prevented Cpx pathway inhibition upon overexpression of a CpxP-β-lactamase (CpxP'-'Bla) translational fusion protein encoded on plasmid pPB. Overexpression of the CpxP'-'Bla fusion from an exogenous promoter confers ampicillin resistance (Amp^R) and down-regulates Cpx-mediated gene expression three to five fold, the same as overexpression of the wild type CpxP protein does (Raivio *et al.*, 1999, Raivio *et al.*, 2000) (Figure 2-2A, compare lanes 1 and 2). Thus, the CpxP'-`Bla protein posesses wild type activity. On lactose-MacConkey indicator media, this inhibition phenotype is manifest as white or Lac$^-$ colonies. We used pPB in combination with a chromosomally encoded Cpx-regulated *cpxP'-lacZ*$^+$ fusion to screen for Lac$^+$ and Amp^R phenotypes, thereby eliminating nonsense mutations that would destroy CpxP function by truncating the protein. pPB was subjected to random mutagenesis by passaging through the *E. coli* mutator strain XL1-Red (Stratagene). Over 10,000 Amp^R colonies, resulting from 20 mutated plasmid pools, were screened on lactose-MacConkey indicator plates, resulting in the isolation of 6 different *cpxP* mutations that yielded Lac$^+$ and Amp^R transformants upon plasmid isolation and retransformation into the reporter strain. Several of these mutations were isolated more than once, indicating that the screen was performed to saturation.

2.3.3 *cpxP* loss-of-function mutations localize to two distinct regions.

Both strands of the mutated pPB plasmids encoding potential loss-of-function CpxP'-'Bla mutants were sequenced to identify the precise mutations. Of the six loss-of-function mutations that we isolated, four of them were transversions and two were transitions in keeping with the broad mutator phenotype of the XL1-Red strain. Five of these mutations altered amino acids between residues 55 and 61 of the unprocessed CpxP and were localized to a single predicted α-helix in the N-terminal region of the protein ($Q_{55}P$, $M_{59}T$, $R_{60}Q$, $D_{61}E$, $D_{61}V$), while the sixth mutation is located at position 128 in the last predicted α-helix in the C-terminal region of CpxP ($Q_{128}H$). Sequence analysis and recloning of the mutant *cpxP* genes showed that no mutations occurred in the *bla* gene, the *tac* promoter, or the plasmid backbone indicating that the observed affects on Cpx-mediated gene expression were due solely to the CpxP amino acid changes.

2.3.4 Loss-of-function CpxP mutants fail to inhibit the Cpx pathway.

In order to determine the extent to which our mutants had lost the ability to inhibit the Cpx response, we transformed the vector control, wild type and mutant pPB plasmids into a strain that contained a chromosomal *cpxP'-lacZ*$^+$ fusion, which allowed for detection of Cpx pathway activity. Activity of the Cpx pathway in the presence of the parent vector, pPB, carrying the wild type CpxP'-'Bla was approximately three to five fold lower than in the presence of either the empty control vector or the six vectors carrying the mutated CpxP'-'Bla fusions (Figure 2-2A, compare lane 2 to lanes 1 and 3 through 8). These results show that all of the mutations impair the ability of CpxP'-'Bla overexpression to inhibit *cpxP'-lacZ*$^+$ expression. To determine if these mutations affected activity of the wild type CpxP protein in the same fashion and rule out the possibility that our mutations exerted their affects in a manner specific to the CpxP'-'Bla fusion protein, we transferred two of the mutations, $D_{61}E$ and $Q_{55}P$, to the previously characterized pCpxP vector, which inhibits the Cpx response via overexpression of the native CpxP protein (Raivio *et al.*, 2000, Raivio *et al.*, 1999). Introduction of the $D_{61}E$ or $Q_{55}P$ mutations into pCpxP resulted in a diminished ability of these plasmids to inhibit *cpxP'-lacZ*$^+$ activity relative to the wild type pCpxP control (Figure 2-2B, compare lanes 3 and 4 to lane 2). Thus, the mutations we isolated exert their effects by altering the inhibitory capacity of the native CpxP protein.

2.3.5 Mutant CpxP'-'Bla proteins are expressed at varied levels.

To examine the expression levels of the mutant compared to the wild type CpxP'-'Bla proteins, whole cell lysates were prepared and immunoblotting was carried out using a monoclonal antibody to β-lactamase. Of the six loss-of-function mutants, $R_{60}Q$, $D_{61}V$, and $D_{61}E$, produced similar or slightly elevated levels of protein of the same size as the wild type CpxP'-'Bla protein (Figure 2-3, compare lane1 with lanes 3, 5, and 9). The other three mutants, $Q_{55}P$, $M_{59}T$, and $Q_{128}H$, exhibited decreased protein expression compared to the wild type CpxP'-'Bla fusion (Figure 2-3, compare lane 1 to lanes 7, 11, and 13). Since sequence analysis and recloning of the mutant *cpxP* genes showed that no mutations in the *bla* gene, *tac* promoter, or plasmid backbone were responsible for the mutant phenotypes (data not shown), these data indicate that the decreased protein levels were due to affects on translation or protein stability.

2.3.6 Diminished mutant CpxP’-‘Bla protein levels are restored by *degP* mutation.

Since the levels of three of the six CpxP’-‘Bla loss-of-function mutants were affected (Figure 2-3), we wondered if CpxP might be controlled by proteolysis. To test this idea, we sought to determine if DegP, a Cpx-regulated protease, affected CpxP’-‘Bla levels. The *cpxP’-‘bla* overexpression plasmid, pPB, and mutated plasmids pPB1 to pPB6 were transformed into a strain carrying a *degP::*Tn10 mutation. Mutation of *degP* resulted in an increase in the level of the wild type CpxP’-‘Bla protein (Figure 2-3, compare lanes 1 and 2). The levels of the three loss-of-function mutant proteins (CpxPQ_{55}P’-‘Bla, CpxPM_{59}T’-‘Bla, and CpxPQ_{128}H’-‘Bla) shown to be present at lower levels than the wild type control (Figure 2-3, lanes 7,11 and 13), were returned to close to wild type levels in the *degP* null strain (Figure 2-3, compare lane 2 to lanes 8, 12 and 15). In addition, the levels of the CpxPD_{61}E’-‘Bla and CpxPD_{61}V’-‘Bla proteins appeared to be slightly increased in the *degP*$^-$ strain background (Figure 2-3, compare lanes 3 and 4, 9 and 10). The levels of a cross reactive protein that served as a convenient loading control were unchanged in either background, as were levels of the periplasmic protein alkaline phosphatase (Figure 2-3, bottom, data not shown). This data suggests that the Q_{55}P, M_{59}T, and Q_{128}H mutant proteins are present at reduced levels due to diminished stability and that DegP affects the stability of both the wild type and mutant CpxP’-‘Bla fusion proteins.

2.3.7 Cpx pathway activity and CpxP’-‘Bla levels are affected by DegP in response to elevated pH.

The above observations suggested that one reason some of the mutant CpxP’-‘Bla fusion proteins lost inhibitory activity was due to a loss of stability that was mediated by DegP-dependent proteolysis. Since we showed that our mutations affect the inhibitory activity of the native CpxP protein in the same fashion (Figure 2-2), we speculated that DegP might influence Cpx signaling through proteolysis of CpxP under inducing conditions. To investigate whether DegP influenced Cpx signaling, we examined the effects of mutating *degP* on Cpx pathway activity and CpxP’-‘Bla levels in the presence and absence of inducing signals (Figure 2-4). To induce the Cpx response, we used alkaline pH, a growth condition shown to induce the Cpx response in both *E. coli* and *S. flexneri* (Danese & Silhavy, 1998, Nakayama & Watanabe, 1995). We grew either wild type or *degP*⁻ strains of *E. coli* bearing a *cpxP'-lacZ*⁺ reporter at pH 5.8, where the Cpx response has been shown to be uninduced, or pH 8.0, where the Cpx response is induced (Danese & Silhavy, 1998) and measured β-galactosidase activity. As previously observed, the Cpx response is strongly induced by growth at pH 8.0, relative to pH 5.8 (Figure 2-4A, compare lanes 1 and 2). In a *degP*⁻ strain, while the pathway was still induced, the level of *cpxP'-lacZ*⁺ expression was diminished almost two-fold (Figure 2-4, compare lanes 1 and 2 to 3 and 4). When we examined the levels of CpxP'-`Bla present in wild type strains at pH's of 5.8 and 8.0, we were unable to detect the fusion protein at a pH of 8.0 (Figure 2-4B, compare lanes 1 and 2). Conversely, in the absence of DegP, the fusion protein was present at approximately equivalent levels at both pH's of 5.8 and 8.0 (Figure 2-4, compare lanes 3 and 4). The levels of a periplasmic control protein, alkaline phosphatase, were unaffected at either pH in wild type or *degP*⁻ strains (Figure 2-4B, bottom). Thus, the ability to induce the Cpx response in a *degP* null is compromised and this correlates with increased levels of the CpxP'-`Bla fusion protein observed under the same conditions.

2.4 Discussion.

In this study, we have taken advantage of a CpxP'-`Bla fusion construct that mediates Cpx pathway inhibition in the same fashion as the native CpxP protein (Figure 2-2) to identify the first essential inhibitory domain in CpxP. We screened over 10, 000 mutagenized plasmids and isolated 5 mutations that localize to a single, highly conserved, predicted α helix in the N-terminus of CpxP (Figure 2-5). The mutations alter the activity of the native CpxP protein in the same fashion (Figure 2-2) and affect either or both inhibitory function and stability (Figure 2-2, 2-3 and 2-4). The reduced levels of some mutant proteins can be restored by mutation of DegP and this lead us to examine the effect of DegP on Cpx signal transduction and CpxP'-`Bla levels. Strikingly, we found that induction of the Cpx response by elevated pH is correlated with disappearance of the CpxP'-`Bla fusion protein and that mutation of *degP* abrogates both these effects (Figure 2-4). Together, the data support a model in which DegP-mediated proteolysis of CpxP relieves inhibition of the Cpx response in the presence of inducing cues (Figure 2-6).

Despite repeated attempts using fusion proteins and immunogenic epitopes as antigens, we have been unsuccessful in raising CpxP-specific antisera. Thus, we cannot be certain at this point that the chromosomally-expressed CpxP is affected in a similar fashion by

DegP in response to inducing cues. However, several lines of evidence suggest that this is the case. Firstly, the mutations we isolated in the CpxP'-'Bla construct affect activity of the native, overexpressed CpxP in the same manner (Figure 2-2). Second, the CpxP'-'Bla construct is dramatically destabilized by the Cpx-specific inducing cue of elevated pH (Figure 2-4B) (Danese & Silhavy, 1998), and this destabilization is DegP-dependent. Finally, mutation of *degP*, which would be expected to stabilize CpxP, leads to diminished Cpx pathway activity in the presence of inducing cues (Figure 2-4A). Thus, although an analysis of the chromosomally-encoded CpxP will be a necessary topic of future studies, these experiments provide strong support for a role of the N-terminus of CpxP as well as DegP in a Cpx signaling cascade (Figure 2-6).

2.4.1 The Cpx envelope stress response is uniquely inhibited by CpxP overexpression.

DiGiuseppe and Silhavy (2003) have recently set forth the hypothesis that CpxP has dual functions as an inhibitory protein and a chaperone, suggesting that CpxP inhibition of the Cpx response upon overexpression may be a by-product of combatting extracytoplasmic protein-mediated toxicity. If this is true then overexpression of other protein folding and degrading factors that belong to the Cpx pathway should also cause a decrease in pathway activity. We overexpressed numerous Cpx-regulated genes, and only when CpxP was overexpressed was there a decrease in pathway activity (Figure 2-1). This supports previous experiments (Raivio *et al.*, 2000, Raivio *et al.*, 1999) and shows that simply overexpressing Cpx-regulated genes does not confer inhibition. These results do not allow us to support or refute the idea that CpxP has dual function, however, they do allow us to speculate that CpxP has a unique function in inhibition of the Cpx response, which none of the other Cpx-regulated protein folding or degrading factors possess.

2.4.2 A predicted N-terminal α-helix plays an essential role in CpxP-mediated inhibition and stability.

In this study we identified two classes of mutations that affect CpxP function and/or stability. Some mutations conferred a loss of inhibition but were otherwise stable (CpxPR$_{60}$Q'-'Bla, CpxPD$_{61}$E'-'Bla, and CpxPD$_{61}$V'-'Bla). Another class of mutations not only conferred loss-of-function but also altered protein stability (CpxPQ$_{55}$P'-'Bla, and CpxPM$_{59}$T'-'Bla) (Figure 2-3 and 2-4). Since most mutations fall in the N terminus, these observations suggest that the N-terminus of CpxP contains a domain that is important both for the inhibition of CpxA via its sensing domain, as well as stabilization of CpxP.

Interestingly, five of the six *cpxP* mutations we identified localized to a span of seven amino acids in a predicted N-terminal α-helix. Since other CpxP homologues show some divergence in the N-terminal region, it has been suggested that the C-terminus is responsible for inhibition and possibly interaction with the highly conserved CpxA (De Wulf *et al.*, 2000). However, the sequence identities of the N-terminal domains from a number of CpxP homologues compared to those of the entire *cpxP* open reading frame (ORF), relative to the *E. coli* CpxP, suggest that this predicted α helix is highly conserved (Figure 2-5). The identities of the predicted N-terminal α helices relative to the entire *cpxP* ORF when compared to those of *E. coli* are 100/88 for *Salmonella spp.*, 100/87.7 for *S. flexneri*, 65/53 for *Yersinia pestis*, 70/46 for *Erwinia carotovara*, and 60/44 for *Photorabdus luminescens* (Figure 2-5A). Even more strikingly, the five mutations that localize to this putative α-helix affect amino acids that are completely conserved between the seven species identified above (Figure 2-5A). This is strong evidence that our mutations are in a critical area of the protein and that this N-terminal α-helix plays an important role in CpxP function. The C-terminal mutation CpxPQ$_{128}$H'-'Bla also falls on a completely conserved amino acid (Figure 2-5B), once again pointing to the importance of these amino acids in the function and stability of CpxP. Helical wheel analysis (Peptool, BioTools Inc.) shows that the N-terminal α-helix is polar except for CpxPL$_{51}$, CpxPL$_{62}$, and CpxPA$_{66}$. This suggests that this helix is most likely located on the outside of the protein and thus one possibility is that our mutations alter a possible protein-protein interaction, such as that which is proposed to occur with the sensing domain of CpxA. It has been suggested that CpxP forms a dimer (D.Issac and T.J. Silhavy, personal communication). Thus, another possibility is that this N-terminal α-helix plays a role in the formation of dimers, and that dimerization is essential for inhibition of the Cpx pathway. Experiments are in progress to differentiate among these hypotheses.

2.4.3 DegP influences both Cpx pathway activity and CpxP'-`Bla levels in response to inducing cues.

Since some mutant CpxP'-'Bla proteins exhibited reduced protein levels (Figure 2-3), we hypothesized that CpxP might be regulated by proteolysis. We reasoned that DegP might be involved in CpxP proteolysis since it is Cpx-regulated (Danese *et al.*, 1995) and has a broad substrate specificity (Jones *et al.*, 2002, Kolmar *et al.*, 1996). We found that, in a *degP* knockout, we were able to recover full length mutant CpxP'-'Bla proteins that were normally undetectable in a wild type strain (Figure 2-3), supporting the idea that CpxP is regulated by proteolysis and that the protease responsible for this degradation is DegP. In order to determine if DegP-dependent proteolysis of CpxP played a role in activation of the Cpx pathway, we analyzed Cpx pathway activity and CpxP'-`Bla levels in wild type and *degP* null strains after exposure to the known Cpx-inducing signal of alkaline pH (Danese & Silhavy, 1998, Nakayama & Watanabe, 1995) and also measured pathway activity when DegP was overexpressed in a wild type background. We found that in alkaline growth media CpxP'-'Bla was undetectable, while at a pH of 5.8 CpxP'-'Bla was stabilized (Figure 2-4). CpxP'-'Bla instability at pH 8 was completely abolished in a *degP* null background (Figure 2-4B), suggesting that DegP was responsible for CpxP'-'Bla degradation in response to elevated pH. The levels of CpxP'-`Bla correlated inversely with induction of the Cpx response. In a wild type strain, growth at pH 8.0 relative to pH 5.8 lead to a 7-8 fold increase in *cpxP'-lacZ*$^+$ reporter activity and this increase was diminished by approximately two-fold in the absence of DegP (Figure 2-4A). When DegP was overexpressed in the wild type background we saw no evidence of increased pathway activity as a result of CpxP degradation (Figure 2-1). We hypothesize the reason why there is not an increase in pathway activity is because CpxP must either be misfolded or in association with misfolded proteins to be degraded by DegP. Together, these data support a model for Cpx signal transduction in which CpxP is rapidly degraded by DegP in the presence of inducing cues, fostering increased pathway activation (Figure 2-6).

It is not surprising that pathway induction is not obliterated in a *degP* null strain, since it has previously been shown that the Cpx response can still be induced in the presence of an overexpressed CpxP fusion protein, albeit to a lower level (DiGiuseppe & Silhavy, 2003). Further, CpxP is not required for Cpx signaling (DiGiuseppe & Silhavy, 2003, Raivio *et al.*, 2000, Raivio *et al.*, 1999). Thus, these results support previous reports that suggest an accessory role for CpxP in the signal transduction scheme. Interestingly, the RseB and TcpH regulators of *E. coli* and *Vibrio cholerae*, respectively, appear to play similar roles. The periplasmic RseB, although not essential for signaling, has been shown to protect the RseA anti-sigma factor from signal-independent proteolysis that would lead to inappropriate induction of the σ^E envelope stress response (Grigorova *et al.*, 2004). Similarly, the periplasmic domain of TcpH appears to be involved in signaling by protecting the membrane-bound regulatory protein TcpP from untimely proteolysis (Beck *et al.*, 2004). We propose that CpxP may serve a similar safeguarding function, protecting the sensing domain of CpxA from unwarranted activation or signaling noise in the absence of veritable envelope stresses.

At this time, we cannot say what event would trigger CpxP proteolysis by DegP in the presence of inducing cues. DegP posesses two PDZ domains (Krojer *et al.*, 2002) that are proposed to bind to substrates and direct them to the proteolytic central cavity of the hexameric protease, although the DegP substrate recognition motif remains undefined. Other PDZ-like domains recognize substrates by virtue of C-terminal recognition motifs bearing hydrophobic determinants (Sonyang *et al.*, 1997). Since the C-terminus of CpxP is predominantly polar/charged, perhaps inducing cues lead to the exposure of an internal domain baring a hydrophobic recognition motif. Alternatively, CpxP may interact with denatured proteins generated by envelope stress, which then target the complex to DegP (Figure 2-6). Whatever the mechanism, the current work identifies the first conserved inhibitory domain in the novel regulator CpxP, implicates DegP-dependent proteolysis in the Cpx signaling cascade, and places CpxP amongst a new class of periplasmic regulatory proteins involved in shielding membrane-bound regulatory proteins from superfluous signaling events. Future studies will be aimed at working out the molecular details of these new facets of Cpx signal transduction.

Table 2-1. Bacterial strains used in chapter 2.

Strains	Description	Reference
MC4100	F$^-$ *araD139* Δ(*argF-lac*)U169 *rspL150* (Str^R) *relA1 flbB5301 decC1 ptsF25 rbsR*	(Casadaban, 1976)
TR50	MC4100 λRS88 (*cpxP'-lacZ*$^+$)	(Raivio & Silhavy, 1999)
TR757	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *degP*::Tn*10*	This study
TR930	TR50 (pBBR1MCS)	This study
TR932	TR50 (pPB)	This study
DB4	TR757 (pPB)	This study
DB8	TR50 (pCpxP)	This study
DB10	TR50 (pPB1)	This study
DB12	TR50 (p*trc99*A)	This study
DB30	TR50 (pPB3)	This study
DB31	TR50 (pPB2)	This study
DB32	TR50 (pPB4)	This study
DB33	TR50 (pPB5)	This study
DB40	TR50 (pPB6)	This study
DB58	TR50 (pDegP)	This study
DB59	TR50 (pDsbA)	This study
DB60	TR50 (pPpiA)	This study
DB61	TR50 (p*trc*99:Spy)	This study
DB70	TR757 (pPB1)	This study
DB71	TR757 (pPB2)	This study
DB72	TR757 (pPB3)	This study
DB73	TR757 (pPB4)	This study
DB74	TR757 (pPB5)	This study
DB75	TR757 (pPB6)	This study
DB115	TR50 (pCpxPU)	This study
DB116	TR50 (pCpxPD$_{61}$EU)	This study
DB117	TR50 (pCpxPQ$_{55}$PU)	This study
DB127	TR50 (pCpxPD$_{61}$E)	This study
DB128	TR50 (pCpxPQ$_{55}$P)	This study

Table 2-2. Plasmids used in chapter 2.

Plasmids	Description	Reference
pBBR1MCS	Expression vector with a multiple cloning site following a *lac* promoter (Cam^R)	(Kovach *et al.*, 1994)
pCpxP	*cpxP* overexpression vector (Amp^R)	(Raivio *et al.*, 1999)
pCpxPD$_{61}$E	*cpxPD$_{61}$E* gene was amplified from pCpxD$_{61}$EU using the CpxP*Kpn* and CpxP*5'Eco* primers and cloned into the Kpn and EcoR1 sites of p*trc99*A (Amp^R)	This study
pCpxPD$_{61}$EU	Site-directed mutagenesis (SDM) was carried out on pCpxPU using the D-61-E1 and D-61-E2 primers;refer to Materials and Methods for complete protocol (Amp^R)	This study
pCpxPQ$_{55}$P	*cpxPQ$_{55}$P* gene was amplified from pCpxPQ$_{55}$PU using the CpxP*Kpn* and CpxP*5'Eco* and cloned into p*trc99*A using the Kpn and EcoR1 sites (Amp^R)	This study
pCpxPQ$_{55}$PU	SDM was carried out on pCpxPU using the Q-55-P1 and Q-55-P2 primers; refer to Materials and Methods for complete protocol (Amp^R)	This study
pCpxPU	*cpxP* gene and flanking upstream region was amplified using CpxP*Bam* and CpxP*Kpn* and cloned into pUC18 BamH1 and Kpn sites (Amp^R)	This study
pDegP	*degP* open reading frame was PCR amplified using the primers DegPKpn and DegPBam and cloned into the Kpn and BamH1 sites in p*trc99*A (Amp^R)	This study
pDsbA	*dsbA* gene was amplified using the DsbA*Kpn* and DsbA*Bam* primers and cloned into the Kpn and BamH1 sites of p*trc99*A (Amp^R)	This study
pPB	CpxP'-'Bla translational fusion overexpression vector; the *cpxP* gene was amplified from MC4100 using CpxP*5Kpn* and CpxPBla primers and clonded into pBBR1MCS using Kpn and BamH1; β-lactamase gene from pUC19 was amplified using BlaCpxP and Bla3*Bam* primers, digested with BamH1 and cloned in-frame downstream of *cpxP* (Amp^R Cam^R)	This study
pPB1	pPB encoding a CpxPD$_{61}$E'-'Bla mutation (Amp^R Cam^R)	This study
pPB2	pPB encoding a CpxPQ$_{55}$P'-'Bla mutation (Amp^R Cam^R)	This study
pPB3	pPB encoding a CpxPR$_{60}$Q'-'Bla mutation (Amp^R Cam^R)	This study
pPB4	pPB encoding a CpxPD$_{61}$V'-'Bla mutation (Amp^R Cam^R)	This study
pPB5	pPB encoding a CpxPM$_{59}$T'-'Bla mutation (Amp^R Cam^R)	This study
pPB6	pPB encoding a CpxPQ$_{128}$H'-'Bla mutation (Amp^R Cam^R)	This study
pPpiA	*ppiA* gene was amplified using PpiA*Kpn* and PpiA*Bam* primers and cloned into the Kpn and BamH1 sites of p*trc99*A (Amp^R)	This study
p*trc99*:Spy	*spy* open reading frame was amplified using the spy*5'Eco* and spy*3'Eco* primers and clonded into p*trc99*A using EcoR1 and BamH1 (Amp^R)	This study
p*trc*99A	High-copy-number expression vector with a multiple cloning site following an IPTG-inducible *trc* promoter (Amp^R)	Pharmacia
pUC18	Cloning vector (Amp^R)	Invitrogen

Table 2-3. Primers used in chapter 2.

Primer	Sequence (5'-3')
Bla*3Bam*	CGG GAT CCT CAC GTT AAG GGA TTT TGG TC
BlaCpxP	CGG GAT CCA CGC TGG TGA AAG TAA AAG A
CpxP*Bam*	CGG GAT CCT GTG CCA GCA AAT AGA GCA G
CpxPBla	CGG GAT CCC TGG GAA CGT GAG TTG CTA C
CpxP*5Kpn*	GGG GTA CCT CGC GAC AGA AAG ATT TTG G
CpxP*Kpn*	GGG GTA CCG GCA AGG AAA ACA GGG TTT A
CpxP*5'Eco*	GGA ATT CCC TCT CTA TCG TTG AAT CGC G
DegP*Bam*	CGG GAT CCG CAC GGC TTA GCA TAA GGA A
DegP*Kpn*	GGG GTA CCC GAA TCT GAA GAA CAC AGC AA
DsbA*Bam*	CGG GAT CCT GAA TAC TCA CGG GCT TTA TG
DsbA*Kpn*	GGG GTA CCG TGG TTA ACC GGG GAA GAT T
D-61-E1	TCA GCA GAT GCG AGA ACT TAT GCA ACA GGC CC
D-61-E2	GGG CCT GTT GCA TAA GTT CTC GCA TCT GCT GA
PpiA*Bam*	CGG GAT CCG TTA CGC CGG GAG AGC AG
PpiA*Kpn*	GGG GTA CCT GAT CGT CAG GTT ACA TAT ATT TCA GA
Q-55-P1	AGT TTA ACC GAA CAT CCG CGT CAG CAG ATG CG
Q-55-P2	CGC ATC TGC TGA CGC GGA TGT TCG GTT AAA CT
spy*3'Bam*	CGG CAT CCC GCA AGG TAG TGG ACA AGA CCG
spy*5'Eco*	GGA ATT CCA ATA ACT GAA AGG AAG GAT ATA G

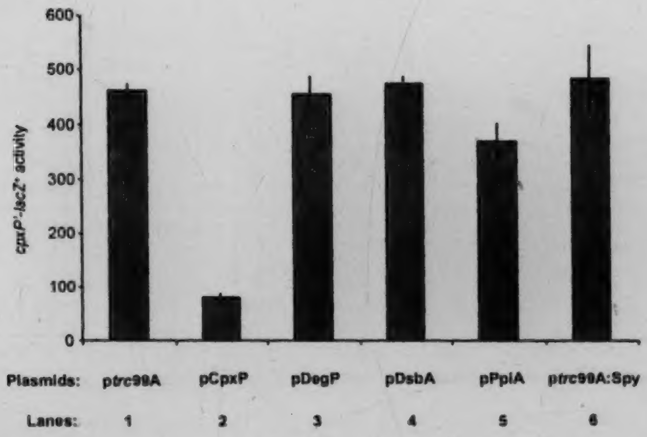

Figure 2-1. Inhibition of the Cpx response is specific to CpxP overexpression. β-galactosidase levels were used to measure the effect of overproduction of specific Cpx-regulated genes on Cpx pathway activity, using strains carrying a *cpxP'-lacZ*$^{+}$ fusion. The strains used were DB12 (lane 1), DB8 (lane 2), DB58 (lane 3), DB59 (lane 4), DB60 (lane 5) and DB61 (lane 6).

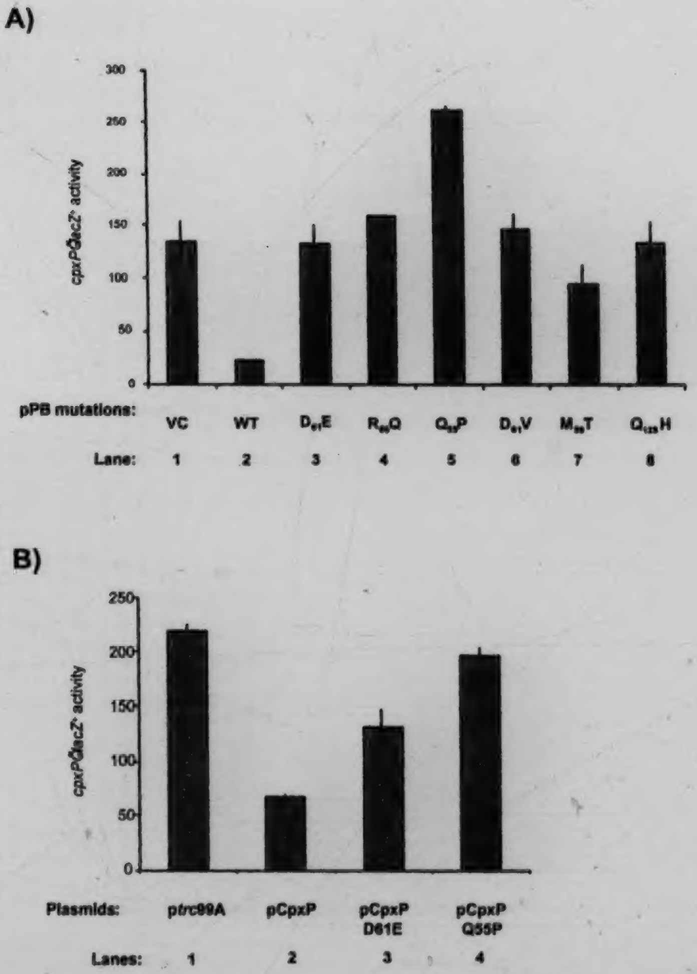

Figure 2-2. The effects of overexpression of LOF CpxP mutants on the Cpx pathway. (A) The effect of overexpression of wild type and mutant CpxP'-'Bla fusions on Cpx-mediated gene expression. β-galactosidase produced from a *cpxP'-lacZ*$^+$ fusion was measured as a reporter of Cpx-mediated gene expression in TR930 (lane 1), TR932 (lane 3), DB10 (lane 3), DB30 (lane 4), DB31 (lane 5), DB32 (lane 6), DB33 (lane 7), and DB40 (lane 8). VC refers to vector control, WT refers to the wild type CpxP'-'Bla protein. (B) The effect of overexpression of wild type and mutant CpxP on *cpxP'-lacZ*$^+$ activity. β-galactosidase production was measured from strain TR50 carrying a chromosomal *cpxP'-lacZ*$^+$ reporter and either p*trc*99A (lane 1), pCpxP (lane 2), pCpxPD$_{61}$E (lane 3) or pCpxPQ$_{55}$P (lane 4).

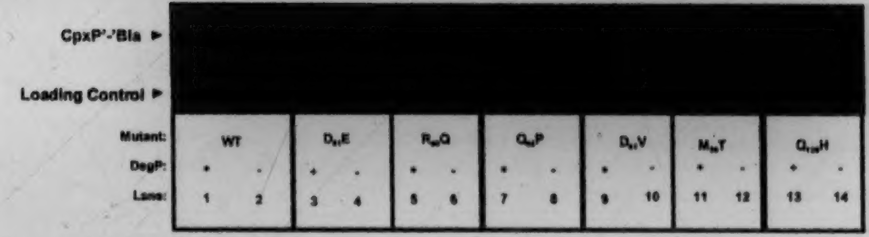
CpxP'-'Bla ►
Loading Control ►
Mutant:
WT
$D_{61}E$
$R_{60}Q$
$Q_{55}P$
$D_{61}V$
$M_{59}T$
$Q_{128}H$
DegP:
+ -
+ -
+ -
+ -
+ -
+ -
+ -
Lane:
1 2
3 4
5 6
7 8
9 10
11 12
13 14

Figure 2-3. Expression levels of wild type and mutant CpxP'-'Bla fusion proteins in wild type and *degP* null strain backgrounds. Western blots were performed on whole cell lysates of wild type (odd lanes) and *degP* null strains (even lanes) transformed with the CpxP'-'Bla encoding plasmid, pPB (TR932 and DB4) (lanes 1 and 2), or the mutated pPB plasmids: CpxPD_{61}E'-'Bla (DB10 and DB70) (lanes 3 and 4), CpxPR_{60}Q'-'Bla (DB30 and DB72) (lanes 5 and 6), CpxPQ_{55}P'-'Bla (DB31 and DB71) (lanes 7 and 8), CpxPD_{61}V'-'Bla (DB32 and DB73) (lanes 9 and 10), CpxPM_{59}T'-'Bla (DB33 and DB74) (lanes 11 and 12), and CpxPQ_{128}H'-'Bla (DB40 and DB75) (lanes 13 and 14) using a monoclonal β-lactamase antibody to label CpxP'-'Bla proteins. WT refers to the wild type CpxP'-'Bla protein. The experiment was performed three times and a representative blot is shown.

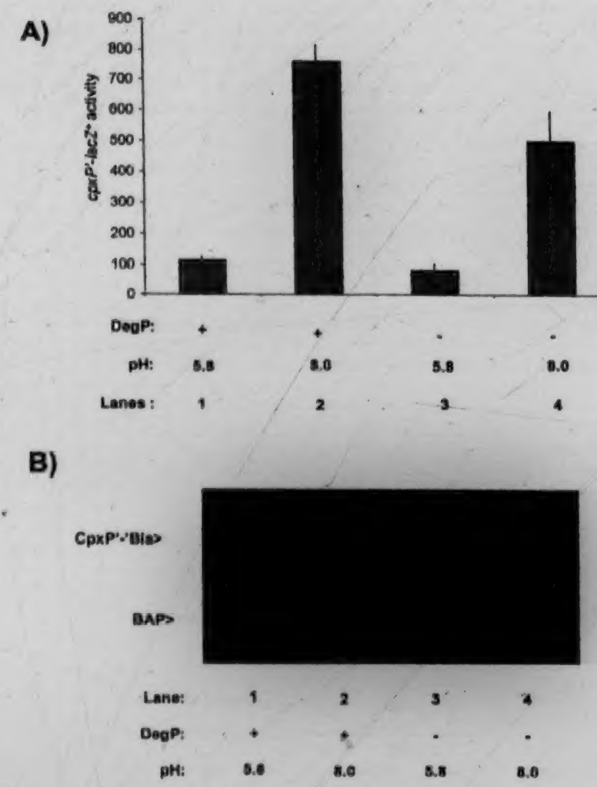

Figure 2-4. Induction of the Cpx pathway and CpxP'-'Bla levels in response to alkaline pH. (A) Induction of the Cpx pathway is reduced in a *degP* null strain. A chromosomal *cpxP'-lacZ*$^+$ fusion was used to determine the effect of pH on Cpx pathway activity in wild type and *degP* null strains. β-galactosidase levels were measured from strains grown in LB buffered with sodium phosphate buffer at a pH of 5.8 (odd lanes) or 8.0 (even lanes). Strains used in this experiment were TR50 (lanes 1 and 3) and TR757 (lanes 2 and 4). (B) CpxP'-`Bla levels are diminished in the presence of Cpx inducing cues in a DegP-dependent fashion. Westerns were carried out on whole cell lysates of wild type (TR932) and *degP* null (DB 4) strains overexpressing a CpxP'-`Bla fusion protein that had been grown in LB buffered with sodium phosphate buffer at a pH of 5.8 (lanes 1 and 3) or 8.0 (lanes 2 and 4). The experiment was performed three times and a representative blot is shown.

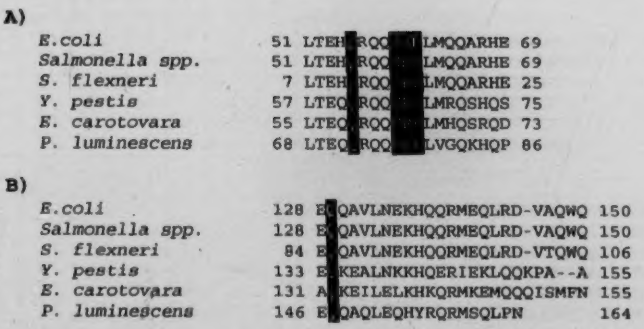
A)
E.coli
51 LTEH RQQ LMQQARHE 69
Salmonella spp.
51 LTEH RQQ LMQQARHE 69
S. flexneri
7 LTEH RQQ LMQQARHE 25
Y. pestis
57 LTEQ RQQ LMRQSHQS 75
E. carotovara
55 LTEQ RQQ LMHQSRQD 73
P. luminescens
68 LTEQ RQQ LVGQKHQP 86
B)
E.coli
128 E QAVLNEKHQQRMEQLRD-VAQWQ 150
Salmonella spp.
128 E QAVLNEKHQQRMEQLRD-VAQWQ 150
S. flexneri
84 E QAVLNEKHQQRMEQLRD-VTQWQ 106
Y. pestis
133 E KEALNKKHQERIEKLQQKPA--A 155
E. carotovara
131 A KEILELKHKQRMKEMQQQISMFN 155
P. luminescens
146 E QAQLEQHYRQRMSQLPN 164

Figure 2-5. Sequence alignment of the full length CpxP proteins from various bacterial species. The amino acid sequence of *E.coli* CpxP was aligned with those of *Salmonella spp.*, *S. flexneri*, *Y. pestis*, *E. carotovara*, and *P. luminscens* using PepTool (Biotools Inc.). The position and extent of each predicted α-helix is indicated, while the locations of the six loss-of-function mutations are indicated in the dark boxes. A) The first predicted N-terminal α-helix pf CpxP, where five of the six mutations fall. B) The predicted C-terminal α-helix of CpxP where a sixth mutation falls.

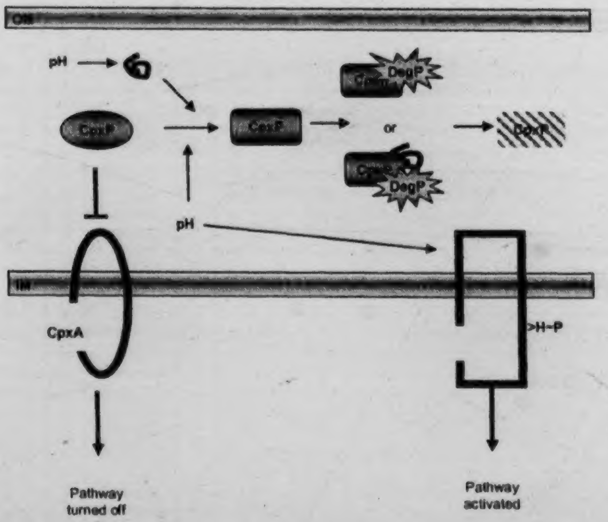
OM
pH
DegP
CpxP
CpxP
or
CpxP
DegP
pH
IM
CpxA
>H-P
Pathway turned off
Pathway activated

Figure 2-6. Model for activation of the Cpx pathway. In the absence of envelope stress CpxP (dark oval) interacts with the sensing domain of CpxA (central region of the periplasmic domain of CpxA), maintaining the pathway in an off state. Inducing cues, such as elevated pH, lead to either or both a conformational change in CpxP (dark rectangle) or its association with misfolded envelope proteins (squiggly line). CpxP or the complex of CpxP with misfolded protein are subsequently degraded by DegP (starburst) and can no longer inhibit CpxA. This leaves the CpxA sensing domain available for maximal induction by cues such as pH. OM=outer membrane, IM=inner membrane, H=histidine, P=phosphate.

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Chapter 3:

The novel signaling protein CpxP is an α-helical dimer that functions with the protease DegP to fine-tune activation and shut-off of the Cpx envelope stress response in *Escherichia coli**

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3.1 Introduction.

The ability to sense a stress in one compartment of the cell and transduce this signal to another is critical to maintain homeostasis and survival. The most widely used way to transduce signals from one compartment to another in nature is via two component signal transduction (TCST) systems, which consist of a histidine kinase (HK) and a response regulator (RR). TCST systems have been identified in all three kingdoms of life, Bacteria, Archaea and Eukarya (Grebe & Stock, 1999, Koretke *et al.*, 2000). However, for Eukarya, they are widespread only outside the animal kingdom with most TCST systems being restricted to plants and free-living organisms such as yeasts, fungi, and protozoa (Thomason & Kay, 2000). Because of this, TCST systems have been targeted for the much needed development of new antimicrobials (Stephenson & Hoch, 2002). How HKs sense regulatory signals is not well understood. It has recently been shown in numerous systems that HKs utilize accessory factors to sense and respond to specific cues (Dartois *et al.*, 1997, Hutchings *et al.*, 2006, Majdalani *et al.*, 2005, Matson and DiRita, 2005, Otto and Silhavy, 2002, Raivio *et al.*, 1999, 2000). Unlocking the mechanism for how HKs sense environmental changes is key to understanding how TCST systems precisely regulate adaptive responses in reaction to the environment.

The Cpx TCST pathway regulates the envelope stress response of *Escherichia coli*. The first studies of the Cpx pathway demonstrated that it was involved in monitoring the integrity of the envelope. It was shown that the pathway is activated in response to over-expression of numerous proteins such as the enteropathogenic *Escherichia coli* (EPEC) type IV bundle forming pilus (BFP) subunit, BfpA (Nevesinjac & Raivio, 2005), the uropathogenic *E. coli* (UPEC) P-pilus subunits, PapG and E, in the absence of their chaperone, PapD (Jones *et al.*, 1997), and a lipoprotein, NlpE (Snyder *et al.*, 1995), as well as alkaline pH (Danese & Silhavy, 1998). Since a number of these proteins are misfolded upon over-expression, it is thought that CpxA senses misfolded or mislocalized proteins within the periplasm. In response to these activating cues there is a phospho-transfer event that takes place between CpxA and CpxR, the RR (Raivio & Silhavy, 1997) to elicit a cellular response. CpxR~P, the phosphorylated response regulator, has enhanced DNA binding ability and is estimated to modulate the activity of over 100 genes (Raivio & Silhavy, 1997, Pogliano *et al.*, 1997, De Wulf *et al.*, 2002). The Cpx regulon contains numerous protein folding and degrading factors such as the periplasmic endoprotease/chaperone, DegP (Danese *et al.*, 1995, Lipinska *et al.*, 1990), and the major disulfide oxidase, DsbA (Bardwell *et al.*, 1991, Danese & Silhavy, 1997, Kamitani *et al.*, 1992, Pogliano *et al.*, 1997) that help clear the envelope of misfolded proteins.

The Cpx pathway has also been implicated in pathogenesis in many bacteria such as *Shigella*, *Salmonella*, *Yersinia*, and *Legionella* species, together with EPEC and UPEC (reviewed by Raivio, 2005). It has been shown to be important in both the transcriptional regulation of major virulence regulatory proteins and to influence early steps in infection. In both *Salmonella* and *Shigella*, the absence of the Cpx pathway affects the expression of *hilA* and *virF*, respectively, which are both involved in invasion functions (Hung *et al.*, 2001, Hernday *et al.*, 2004, Nakayama *et al.*, 2003, Nakayama & Watanabe, 1995, Nakayama & Watanabe, 1998). CpxR~P has been shown to bind to a region that controls *pap* phase variation and activates P pilus expression (Hung *et al.*, 2001). As well, the Cpx pathway has been shown to shut down type III secretion (TTS) in both *Yersinia* and EPEC (Carlsson *et al.*, 2007, MacRitchie *et al.*, 2007). The affects the Cpx pathway has on TTS have been shown to be both transcriptional (MacRitchie *et al.*, 2007, Carlsson *et al.*, 2007) and post-transcriptional (MacRitchie *et al.*, 2007). In addition the Cpx pathway has been shown to be involved in the assembly of at least two types of pili, the P pilus of UPEC and the type IV bundle-forming pilus (BFP) of EPEC (Hung *et al.*, 2001, Nevesinjac & Raivio, 2005). In strains where CpxR is absent the P pili that are elaborated are considerably shorter than those of a wild type strain (Hung *et al.*, 2001).. In an EPEC *cpxR* mutant, there is a decreased level of auto-aggregation and adherence to eukaryotic cells, which are attributable to a decrease in BFP levels (Nevesinjac & Raivio, 2005). Since the Cpx response is up-regulated by the production of misfolded pilus subunits, it has been hypothesized that Cpx pathway induction by misfolded proteins generated during pilus assembly leads to the up-regulation of DsbA and DegP, which are key players in the biogenesis and assembly of pili (Jones *et al.*, 1997, Donnenberg *et al.*, 1997).

An interesting aspect of the Cpx pathway is the presence of a third component, CpxP, which acts as an accessory signal transduction protein. Initial characterization of CpxP demonstrated that it serves as an inhibitor protein. Over-expression of CpxP causes a three to five fold decrease in Cpx pathway activity that is dependent on the CpxA HK (Buelow & Raivio, 2005, Raivio *et al.*, 2000, Raivio *et al.*, 1999). It has been shown that a highly conserved N-terminal domain within CpxP is important for this inhibitory affect, as five loss-of-function mutations have been isolated within this region (Buelow & Raivio, 2005). It is hypothesized that under non-stressful conditions CpxP interacts with the sensing domain of CpxA, inhibiting its autokinase activity (Fleischer *et al.*, 2007, Raivio &

Silhavy, 1997) and maintaining the pathway in an off state. Upon activation, CpxP is thought to be titrated away from the CpxA sensing domain. Isaac *et al.* (2005) provided evidence that suggests this might occur by binding of CpxP to misfolded proteins, which it then targets together with itself to the protease, DegP. It is also possible that envelope stress simply leads to CpxP misfolding which then targets it for degradation by DegP. In either case, CpxP is degraded by DegP (Buelow & Raivio, 2005, Isaac *et al.*, 2005), leaving the sensing domain of CpxA free to sense the stress at hand and elicit the appropriate response. In the presence of CpxP over-expression or in the absence of CpxP, the pathway can still be activated (DiGiuseppe & Silhavy, 2003, Raivio *et al.*, 1999). This has led to the hypothesis that CpxP is important in shutting off the pathway once envelope stress has been relieved. The physiological role of CpxP has remained enigmatic since the majority of studies to date have involved artificial conditions in which CpxP or misfolded proteins are over-expressed from plasmids.

In this study we tested various aspects of the current model by analyzing CpxP stability in the presence of a variety of inducing cues, examining Cpx signal transduction in the presence of *cpxP* and/or *degP* null mutations, and performing biochemical analyses of CpxP. CpxP half-life studies revealed that inducing cues of the Cpx pathway can be divided into those that cause CpxP proteolysis and those that do not, suggesting that there are multiple signaling inputs that control Cpx pathway activity. Surprisingly, we found that CpxP is not only important in the inhibition of the Cpx pathway, but also plays a role in the activation of the pathway. We showed that DegP is also needed for the proper activation of the Cpx pathway and that it functions upstream of CpxP. In addition we began to analyze the structure of CpxP in order to gain insight into the signal transduction and protein folding roles of this novel protein. We show that it is a dimer both *in vivo* and *in vitro* and undergoes a conformation change in response to the inducing cue, alkaline pH.

3.2 Material and Methods.

3.2.1 Bacterial strains and plasmids.

Strains and plasmids used in this study are listed in Table 3-1 and 3-2. All strains were made using standard genetic techniques (Silhavy *et al.*, 1984).

3.2.2 Media, antibiotics and growth conditions.

Strains were grown on Luria-Bertani (LB) agar (Silhavy *et al.*, 1984) at 30°C or 37°C. All liquid cultures were grown in LB broth with aeration at either 30°C or 37°C. Strains were maintained with the appropriate selection, 100 μg/mL ampicillin (Amp), 25 μg/mL chloramphenicol (Cam), 25 μg/mL Kanamycin (Kn) or 25 μg/mL tetracycline (Tet). All antibiotics were purchased from Sigma.

3.2.3 Western blot analysis.

Strains were grown overnight at 30°C with aeration. The next day overnight cultures were diluted 1:50 into 5 mL of LB with the appropriate antibiotic and buffered with 1M Na-phosphate buffer at the indicated pH (Sambrook *et al.*, 1989) to a final concentration of 100mM to either induce or repress the Cpx pathway. Cultures were then grown at 37°C for 4 hours, and the O.D.$_{600}$'s of the cultures were measured. Whole cell extracts were prepared by pelleting volumes that were adjusted to correspond to 1mL of the culture that reached the lowest O.D.$_{600}$ and resuspended in 50μL of 2X SDS-PAGE loading dye (Raivio *et al.*, 1999). Lysates were prepared, electrophoresed and transferred to nitrocellulose as previously described by Buelow and Raivio (2005). The membrane was labelled as previously described by Raivio *et al.* (1999) with β-lactamase monoclonal antibody (1:1 000 dilution) (QED BioScience Inc.). A secondary anti-mouse antibody conjugated to alkaline phosphatase (Sigma) was used at a 1:25 000 dilution. Proteins were visualized using the Immun-Star™ AP substrate pack chemiluminescent kit (BioRad).

3.2.4 Adsorption of non-specific antibodies from anti-CpxP serum.

Anti-CpxP serum was collected previously by Dr. Tracy L. Raivio from rabbits that had been injected with purified MBP-CpxP protein. This anti-CpxP serum was optimized as previously described (Miller & Shakes, 1995). Briefly, overnight cultures of DB223 were sub-cultured 1:50 into 250mL of fresh LB and grown at 37°C to late log phase. Bacteria were harvested by centrifugation and resuspended in 1/10th of the original volume of 0.9% NaCl. The suspension was frozen on dry ice and thawed on wet ice. The slurry was then sonicated 5 times for 30 seconds and incubated with 5 times the volume of -20°C acetone for 30 minutes on ice. The acetone slurry was centrifuged at 9000g for 20 minutes at 4°C and resuspended in fresh -20°C acetone to the same volume before centrifugation. Centrifugation was repeated and the pellet was spread on filter paper to dry overnight. The next day the acetone powder was collected and ground into a fine powder. 25mg of acetone powder per milliliter of anti-sera was mixed with the anti-serum and mixed for 1 hour at room temperature on a Labquake (Fisher) to adsorb non-specific antibodies. The anti-serum slurry was then centrifuged for 5 minutes at top speed in a table top microcentrifuge (Eppendorf) and the supernatant was drawn off. The cleaned anti-serum was tested via Western blot analysis to determine relative purity. Normally, the anti-serum was put through two or three cycles of adsorption to remove most of the unwanted cross reactive bands.

3.2.5 Protein stability assays.

Bacteria were grown overnight in media with appropriate antibiotics at 30°C. For strains carrying derivatives of the pBAD-Kn vector, 0.2% glucose was added with the appropriate antibiotics to overnight cultures. Overnight cultures were diluted 1:50 into 20mL of fresh LB with appropriate antibiotics and inducers and grown at 37°C to mid-log phase (O.D.$_{600}$ of 0.6). Strains carrying ptrc99A derivatives were induced with 1mM isopropylthio-β-D-galactoside (IPTG), and pBAD-Kn derivates were induced with 0.2% arabinose. Induction or repression of the Cpx response by pH was accomplished by adding 1M sodium phosphate buffer at the appropriate pH (Sambrook *et al.*, 1989) to the media to a final concentration of 100mM. 20mg/ml spectinomycin was added to a final concentration of 0.5mg/mL to inhibit further protein synthesis (Aldridge *et al.*, 2003). 1mL whole cell lystates for strains expressing the CpxP'-'Bla protein were collected at 0, 1, 2, 5, 10, 15, 20, 25, and 30 minutes after addition of spectinomycin, centrifuged and resuspended in 50μL of 2X SDS-PAGE loading dye (Raivio *et al.*, 1999). Samples were analyzed via western blot, as described above. β-lactamase monoclonal antibody (1:1 000 dilution)(QED BioScience Inc.) was used as the primary antibodies. A secondary anti-mouse antibody conjugated to alkaline phosphatase (Sigma) was used at a 1:25 000 dilution. Protein concentration was quantified via band intensity using the AlphaEase software package and a FluorChem IS-5500 imaging system (AlphaInnotech, Fisher Scientific). The densities at each time point were measured using the spot density function. Protein density was then graphed versus time and protein half-life was determined. Each assay was performed in either duplicate or triplicate. Half-life range represents either average difference or standard deviation.

3.2.6 Construction of *cpxP* and *degP* mutant strains.

The *cpxP*::FRTKn and *degP*::FRTKn allele from the Keio *E.coli* K-12 knock-out library (Baba *et al.*, 2006) was transduced into MC4100 resulting in DB266 and DB260, respectively. pFlp2 (Hoang *et al.*, 1998) was then transformed into the resulting strain. Transformants were recovered for 1 hour at 30°C and then plated onto LB Amp plates and grown overnight at 37°C. Deletion of the chromosomally integrated FRTKn markers was determined by patching transformants in duplicate onto LB and LB Kn plates. After overnight growth, 4 colonies that exhibited Kn^S phenotypes were grown up in 2 mL of LB for 4 hours, at which time they were serially diluted (10^{-2} to 10^{-4}) and plated on NaCl free LB Amp plates supplemented with 5% sucrose to select against pFlp2. The 10^{-4} dilution was also plated on normal LB plates as a control. After growth at room temperature for 2 days, sucrose supplemented plates showed a 3 to 5 order of magnitude decrease in colony number compared to the control plates. Colonies from the sucrose supplemented plates were then patched onto LB and LB Amp plates. Those that exhibited Amp^S after overnight growth were genotyped by PCR, to confirm that *cpxP* or *degP* had been deleted.

A *cpxP::kan* allele was created by amplifying the Kan^R cassette from pUC4K using KanBgl-forward (5'-GAA GAT CTG TTT TCC CAG TCA CGA CGT T-3') and KanBgl-reverse (5'-GAA GAT CTT GTG GAA TTG TGA GCG GAT A-3') and inserting it into the BglII (Invitrogen) site of *cpxP* found in pCpxPU (Buelow & Raivio, 2005), resulting in pCpxPKn. *cpxP::kan* was amplified from pCpxPKn using the CpxP5'Kpn (5'-GGG GTA CCT CGC GAC AGA AAG ATT TTG G-3') and CpxPBla (5'-CGG GAT CCC TGG GAA CGT GAG TTG CTA C-3') primers, digested with KpnI and BamH1 (Invitrogen) and cloned into pMAK705 (Hamilton *et al.*, 1989), resulting in pMAK705-CpxPKn. pMAK705-CpxPKn was used to transfer the *cpxP*::kan allele onto the chromosome using the allelic exchange protocol of Hamilton *et al.* (1989).

3.2.7 Monitoring Cpx pathway turn-on and shut-off using a cpxP'-lux$^+$ reporter.

Strains were grown overnight with the appropriate antibiotics in LB buffered at a pH of 5.8 with 100mM sodium phosphate (Sambrook *et al.*, 1989). The next day cells were subcultured 1:50 in 50ml of LB buffered at either a pH of 5.8 for 1 hour for turn on experiments or at a pH of 8.0 for 2 hours for shut off experiments at 37°C with aeration. Cells were centrifuged, washed in normal LB and then resuspended in LB buffered at a pH of 8.0 (turn on) or 5.8 (shut off). Cpx pathway induction was measured by monitoring light production from a *cpxP'-lux*$^+$ transcriptional reporter every 5 minutes for 1 hour (turn on) and every 15 minutes until a plateau in light production was reached (shut off) using the Wallac Victor^2 plate reader (PerkinElmer). Fold induction of the Cpx pathway was determined by comparing pathway activity at a given time to the zero time point for each strain. Experiments were performed in triplicate. Error bars represent standard deviation.

3.2.8 Formaldehyde mediated *in vivo* cross-linking.

Cross-linking experiments were performed as described in Larsen *et al.* (Larsen *et al.*, 1994), with minimal changes. Cells were subcultured 1:50 in 5mL fresh media with the corresponding antibiotics and grown until mid log phase (O.D.$_{600}$ = 0.6). Cells were harvested and resuspended in the original volume of 0.1M sodium phosphate buffer (pH 6.8). 50 μL of formaldehyde (37% W/W)(Fisher) was added to 1mL sample aliquots. Samples were mixed at room temperature using a Labquake mixer (Fisher) for either 10 or 15 minutes, centrifuged and resuspended in 50 μL of 2X SDS-PAGE loading buffer. Samples were analyzed via western blot analysis (described above). The primary antibody used was the CpxP polyclonal antibody (1:25 000) and the secondary antibody was an anti-rabbit antisera conjugated to alkaline phosphatase (1:25 000) (Sigma).

For cross-linking of CpxP loss-of-function mutants, culture O.D.$_{600}$ was measured and volumes taken for analysis were standardized to the lowest O.D.$_{600}$. Cells were harvested, cross-linked and analyzed as described above. To determine the dimer:monomer ratio, protein density of the monomer and dimer bands were determined using the AlphaEase software package and a FluorChem IS-5500 imaging system (AlphaInnotech, Fisher Scientific) and the density of the dimer band was then divided by the density of the monomer band. Experiments were performed in triplicate. Error bars represent standard deviations.

3.2.9 Expression and purification of wild type CpxP for biochemical assays.

Wild type CpxP was over-expressed as an MBP fusion from pMCP in TR757, a *degP* null strain (SS1) (Table 3-1 and 3-2). Cells containing the pMCP plasmid (Table 3-1 and 3-2) were grown at 30°C in LB media with 2 g/L glucose and ampicillin until they reached an O.D.$_{600}$ of 0.7 at which point they were induced with 0.2 mM IPTG and grown overnight at 22°C. Cells were then harvested, resuspended and osmotically shocked to release the periplasmic fusion protein as per the pMAL^{TM} protein fusion and purification system manual (New England Biolabs). The shock fluid was then applied to amylose resin (New England Biolabs) which was pre-equilibrated with wash buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). After loading, the column was washed with 10 volumes of wash buffer and then the CpxP-MBP fusion protein was eluted with elution buffer (wash buffer + 10 mM final concentration maltose). The fusion was then buffer exchanged into 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$, and concentrated to approximately 2 mL using an Amicon Ultra-15 10 K MWCO spin concentrator (Millipore, Fisher Scientific). The concentrated sample was quantitated using the theoretical extinction coefficient at 280 nm of 78840 $M^{-1}cm^{-1}$ calculated using ProtParam (Gasteiger *et al.*, 2005) from the primary sequence of CpxP-MBP. Factor Xa protease (GE Healthcare) was then added to 2.5% w/w ratio Factor Xa:CpxP-MBP and incubated for 16 h at 4°C. The cleavage reaction was monitored by SDS-PAGE and the reaction was stopped with phenylmethylsulfonyl fluoride (Sigma). The cleavage reaction was then diluted up in wash buffer and re-flowed over the amylose resin to separate CpxP from MBP and residual fusion protein. The CpxP-containing flow through was then concentrated using an Amicon Ultra-15 5 K MWCO spin concentrator (Millipore, Fisher Scientific) and applied to a Superdex^{TM} 75 26/60 gel filtration column (GE Healthcare) which was equilibrated in buffer containing 250 mM NaCl, 1 mM EDTA and 50 mM sodium phosphate (pH 5.8 or 8.0). The purified CpxP sample was subjected to MALDI-TOF mass spectrometry (University of Alberta Chemistry Mass Spectrometry Facility), where it was found to have an average m/z of 17336 ± 5 Da, which corresponded to the protein having its last two C-terminal amino acids removed. Amino acid analysis (Alberta Peptide Institute) produced an experimental extinction coefficient at 280 nm of 12214 $M^{-1}cm^{-1}$ which was subsequently used for protein quantitations.

3.2.10 Size-exclusion chromatography with Multi-angle Laser Light Scattering (MALLS).

50 µL of a 2 mg/mL CpxP sample was injected at 0.5 mL/minute onto a Superose^{TM} 6 HR 10/300 gel filtration column (GE Healthcare) which was equilibrated with 50 mM sodium phosphate (pH 5.8 or 8.0), 250 mM NaCl, and 1 mM EDTA. After flowing over the column, the effluent was directly passed over an in-line DAWN EOS^{TM} MALLS, an Optilab rEX^{TM} differential refractive index detector and an in-line Wyatt quasi-elastic light scattering (QELS) instrument (Wyatt Technologies, Santa Barbara, CA). Light scattering data was processed using the ASTRA version 4.90 software. Averages of molecular weights and

radii of hydration (R_H) were calculated from the elution peak (refer to (Lu *et al.*, 2006). A minimum of two runs per pH were collected from which the average and standard deviation were determined.

3.2.11 Small angle X-ray analysis (SAXS).

Small-angle X-ray scattering curves were collected on Beamline 12.3.1 at the Advance Light Source, at the Lawrence Berkeley National Laboratory in Berkeley, California U.S.A. Data were collected on wildtype CpxP at ~ 7.5 mg/mL dialyzed against 250 mM NaCl, 50 mM sodium phosphate (at either pH 5.8 or 8.0), 1 mM EDTA, 10% glycerol. CpxP exhibited no radiation-induced damage as determined by comparison of a series of exposure times (two 6 s, a 60 s and a final 6 s exposure, data not shown). Guinier plots plotted in PRIMUS were linear in the range $s.Rg < 1.3$, an approximation that holds true only for non-aggregated samples. The radii of gyration (*Rg*) calculated from the Guinier plots were 23.5 ± 0.1 and 22.3 ± 0.1 Å for the proteins at pH 5.8 and 8.0 respectively. The particle-probability distribution plot, a transformation from reciprocal to real-space, calculated using the program GNOM gave mean estimates of the maximum particle dimension (D_{max}) for all four exposures in the series of 66 and 60 Å for the pH 5.8 and 8.0 samples with corresponding *Rg* of 22.9 and 21.9 Å. The program GASBOR was used to generate low-resolution *ab initio* envelopes of wildtype CpxP at each pH (Figure 3-4C). Each envelope was constructed from 15 GASBOR calculations, superimposed and averaged with DAMAVER and filtered with DAMFILT. GASBOR envelopes were calculated in three ways: with no enforced symmetry having either 149 (assumed monomer) or 298 (assumed dimer) amino acids and with P2 symmetry, 298 amino acids.

3.2.12 Circular dichroism.

CD experiments were performed on a Jasco J720 spectropolarimeter. Far-UV (255-190 nm) experiments used 0.25 mg/mL CpxP samples in a thermostated fused silica cell with a path length of 0.05 cm at 24°C. For secondary structure analysis, spectra were collected with CpxP samples in 50 mM sodium phosphate pH (5.8 or 8.0) to reduce unwanted signal contributions from NaCl and EDTA at wavelengths below 200 nm. Spectra in this buffer were the same as spectra containing 250 mM NaCl and 1 mM EDTA. Raw ellipticities were subtracted from the buffer and converted into mean residue ellipticities using the average amino acid weight of 117.1 Da, calculated from the CpxP primary sequence. Secondary structure predictions were performed using the DICHROWEB online server

(Whitmore & Wallace, 2004). Several algorithms with reference datasets 4 and 7 were compared and those which gave a normalized root mean square deviation (NRMSD) < 0.1 were used to calculate the fractions of secondary structure. These algorithms were CONTIN/LL (Provencher & Glockner, 1981) and CDSSTR (Johnson, 1999) For melting temperature (T_m) experiments, ellipticity was recorded at 220 nm for 5 minutes at each temperature, measuring a reading every second. From the data, an average ellipticity at each temperature was calculated. CpxP samples were in 250 mM NaCl, 1 mM EDTA, and 50 mM sodium phosphate (pH 5.8 or 8.0), and were at a concentration of 0.25 mg/mL in a 0.05 cm silica cell. The samples were equilibrated for 5 minutes at each temperature before data collection. Temperatures measured ranged from 24 to 62°C, employing a Lauda water bath (Brinkmann Instruments) to control the temperature of the cell. The loss of ellipticity at 220 nm indicated unfolding of CpxP and T_m is defined as being the temperature at which the ellipticity at 220 nm decreases by half. Melting temperatures were calculated using the SigmaPlot 2001 software (SPSS Inc.). T_m values represent an average of two experiments. Near-UV (320-255 nm) experiments used 1 mg/mL samples of CpxP in a 1 cm path length thermostated fused silica cell at 24°C. CpxP samples were in 50 mM sodium phospate pH (5.8 or 8.0), 250 mM NaCl, 1 mM EDTA. Ellipticity data was converted to mean residue ellipiticity as described above. Both far-UV and near-UV spectra were an average of 12 scans.

3.3 Results.

3.3.1 CpxP'-'Bla stability is affected differently by DegP depending on the inducing cue.

When the Cpx response is activated, both DegP and CpxP are produced at elevated levels, however CpxP does not function as an inhibitor under these conditions (DiGiuseppe & Silhavy, 2003, Buelow & Raivio, 2005). Further, it was previously shown that CpxP is a substrate for the DegP protease (Buelow & Raivio, 2005, Isaac *et al.*, 2005). Under the Cpx-inducing condition of either alkaline pH or over-expression of the misfolded pilus subunits PapG or PapE, CpxP is absent and its absence is dependent on DegP (Isaac *et al.*, 2005, Buelow & Raivio, 2005). This suggests that DegP affects CpxP stability in the presence of Cpx inducing cues and that the destabilization of CpxP prevents it from acting as an inhibitor of the Cpx response. To test this model, we determined the half-life of a functional CpxP'-'β-lactamase (CpxP'-'Bla) fusion protein (Buelow & Raivio, 2005) under non-inducing conditions as well as in the presence of conditions that are known to induce the Cpx pathway.

Initially we performed control experiments to verify that our CpxP'-'Bla fusion protein behaved in a similar fashion to the native CpxP. It was determined that under non-inducing conditions CpxP'-'Bla had a half-life of approximately 10 minutes ± 2 min (Table 3-3, row 1). To ensure that the CpxP'-'Bla half-life observed reflected that of CpxP and not that of β-lactamase (Bla) or that of a general stress response due to the addition of spectinomycin during the half-life assays, we determined the half-life of Bla and bacterial alkaline phosphatase (BAP). The half-life of Bla was greater than 30 min (Table 3-3, row 16), indicating that the half-life value observed for CpxP'-'Bla did not simply reflect that of Bla. Similarly the half-life of BAP was greater then 30 minutes (Table 3-3, row 17). We also showed that addition of spectinomycin had no affect on Cpx pathway activity (data not shown). The half-life of CpxP'-'Bla in the absence of DegP was greater than 30 min (Table 3-3, row 7), demonstrating that DegP is involved in CpxP'-'Bla turnover in the same fashion as it controls turnover of the native CpxP protein (Isaac *et al.*, 2005, Buelow & Raivio, 2005).

Next, we examined CpxP'-'Bla stability in the presence of a variety of Cpx inducing cues. Previous studies demonstrated that steady-state levels of CpxP were diminished in the presence of over-expression of the UPEC P pilus subunits PapE and PapG, but not in the presence of NlpE (Isaac *et al.* 2005), suggesting that CpxP proteolysis may not be a general feature of Cpx pathway induction. However, since these experiments did not examine CpxP stability per se, we wished to test this idea by determining CpxP'-'Bla half-lives in the presence and absence of a representative set of Cpx activating signals. We first examined the inducing cue of alkaline pH. Danese and Silhavy (1998) showed that at a pH of 5.8, the Cpx pathway is repressed, however at pHs of 7.8 and 8, the pathway is induced. When we grew bacteria carrying the CpxP'-'Bla fusion protein at a pH of 5.8, CpxP'-'Bla had a half-life of greater than 30 min (Table 3-3, row 4), indicating increased protein stability relative to uninduced conditions (pH 7.0). However, when the cells were grown in media at a pH of 7.8 or 8, CpxP'-'Bla was undetectable (Table 3-3, rows 5 and 6), indicating decreased stability. To determine if the decreased stability of CpxP'-'Bla at pHs of 7.8 and 8 was dependent on DegP, we grew *degP* null strains expressing the CpxP'-'Bla fusion protein in media at pHs of 7.8 and 8. Under these conditions, the half-life of CpxP'-'Bla was greater than 30 min (Table 3-3, rows 8 and 9), indicating that, as expected, DegP is responsible for CpxP'-'Bla degradation at elevated pHs.

To determine if DegP dependent degradation of CpxP was a general feature of Cpx pathway induction we looked at the half-life of CpxP'-'Bla under two other known inducing conditions, overproduction of NlpE (Snyder *et al.*, 1995) and overproduction of the unprocessed major subunit of the typeIV BFP of EPEC, pre-bundlin or BfpA (Nevesinjac & Raivio, 2005). We over-expressed NlpE from a pBAD-Kn plasmid that was induced by 0.2% arabinose and BfpA from a ptrc99A plasmid that was induced with 1mM IPTG (Stone *et al.*, 1996). To ensure that the inducers, arabinose and IPTG, and also the pBAD-Kn and ptrc99A plasmids, did not affect CpxP'-'Bla stability, we looked at the half-life of CpxP'-'Bla in the vector control strains grown in the presence of the appropriate inducer. The half lives of CpxP'-'Bla in the pBAD-Kn and ptrc99A backgrounds were 9 min ± 9 sec and 8 min ± 32 sec, respectively (Table 3-3, rows 10 and 12). Since these half-life values are comparable to those of the wild type CpxP'-'Bla, we can conclude that any changes in half-life value of CpxP'-'Bla in response to over-expression of either NlpE or BfpA are not due to the vector or the inducers. When NlpE was over-expressed, CpxP'-'Bla had a half-life of 7 min and 37 sec ± 3 sec (Table 3-3, row 11). The half-life of CpxP'-'Bla when NlpE was over-expressed is within error of that of the wild type fusion protein, suggesting there is no difference in CpxP stability between these two conditions. The half-life of CpxP'-'Bla when pre-bundlin, BfpA, was over-expressed was not calculated since no protein was detected under these conditions (Table 3-3, row 13). However in a *degP* null the half-life of CpxP'-'Bla in the presence of BfpA over-expression was increased to greater than 30 min (Table 3-3, row 15). Therefore, like elevated pH and over-expression of PapG and PapE, over-expression of BfpA leads to a decrease in CpxP'-'Bla stability that is DegP-dependent. These results demonstrate that CpxP stability is differentially affected by DegP depending on the activating signal.

3.3.2 CpxP and DegP are required for the rapid induction and shut-off of the Cpx response and DegP functions upstream of CpxP.

Characterization of CpxP has relied on experiments in which CpxP and/or misfolded proteins have been over-expressed (Buelow & Raivio, 2005, Raivio *et al.*, 2000, Raivio *et al.*, 1999, Isaac *et al.*, 2005). Although these experiments have defined CpxP as an inhibitor of the Cpx pathway and possibly a proteolytic adaptor protein, they have not addressed the role of CpxP under physiological conditions. Thus, we sought to identify previously unobserved phenotypes attributable to CpxP by studying Cpx signal transduction in wild type and *cpxP* null strain backgrounds. We hypothesized that since

CpxP has been characterized as an inhibitor of the Cpx pathway, a *cpxP* null strain may be defective in shutting off the pathway after induction. To measure the activation and repression of the pathway we used a *cpxP'-lux*$^+$ transcriptional fusion. This reporter is regulated as expected by Cpx inducing cues (Figure 3-1B, Price and Raivio, in preparation, data not shown). Strains containing the plasmid encoding the *cpxP'-lux*$^+$ reporter to measure Cpx pathway activity or the respective vector control were initially grown at pH 8.0 for 2 hours to activate the pathway and then shifted to a pH 5.8. Luminescence was measured every 5 minutes for the first hour and then every 15 minutes until a plateau was reached. In strains carrying the vector control the relative levels of luciferase were insignificant (data not shown). In the wild type strain it took 100 minutes $\pm$ 8.66 minutes to reach maximum shut off (Figure 3-1A). In contrast it took the Δ*cpxP* strain 150 $\pm$ 0 minutes to reach maximum shut off (Figure 3-1A). Even though it took the Δ*cpxP* strain 1.5 times longer to reach maximum shut off, the overall fold shut off of the pathway was comparable in the wild type (3.82 $\pm$ 0.84) and the Δ*cpxP* strains (4.76 $\pm$ 2.10) (Figure 3-1A). These observations demonstrate that, as hypothesized, CpxP plays an important role in restoring basal level activity of the Cpx TCST system upon relief of envelope stress.

To study induction of the pathway, the same strains as above were initially grown at a pH of 5.8, to ensure that the pathway was repressed and then shifted to a pH of 8.0. Pathway activity was measured every 5 minutes and fold induction was determined by comparing each time point to time zero for each strain. In the strains carrying the vector controls, the relative level of luciferase activity was negligible (data not shown). As observed previously (Danese & Silhavy, 1998), the activity of the *cpxP'-lux*$^+$ reporter increased over time after the up shift to pH 8.0 (Figure 3-1B). In the Δ*cpxP* strain, while the pathway was still induced, the fold induction of *cpxP'-lux*$^+$ expression was about four fold below that of the wild type strain during the initial induction period (Figure 3-1B, compare the wild type and Δ*cpxP* strains at 10, 15, 20, 25 and 30 minutes). By 35 minutes after induction the levels of induction by both strains were comparable, although the fold activation of the Cpx response in the Δ*cpxP* strain remained slightly below that of the wild type strain (Figure 3-1B, Figure 3-2). This data suggests that, surprisingly, the inhibitor CpxP plays a role in the early activation of the Cpx pathway. Together, the data support a model in which CpxP is important for efficient activation and shut-off of the pathway.

We previously showed that DegP was responsible for the degradation of CpxP'-'Bla and CpxP in the presence of Cpx inducing cues (Buelow & Raivio, 2005) (Table 3-1 and 3-2, Figure 3-1). This suggests that DegP functions upstream of CpxP in the control of Cpx TCST. We sought to test this model by determining the epistasis of DegP relative to CpxP. We performed these experiments by growing wild type, *cpxP* null, *degP* null or *cpxP degP* double null strains carrying a *cpxP'-lux*$^+$ reporter construct initially at pH 5.8 to ensure the pathway was turned off and then shifted the strains to pH 8.0. Luciferase activity was measured every 5 minutes for the first 75 minutes and then every 15 minutes to 105 minutes. Mutation of *degP* resulted in an overall decrease in the fold induction of the pathway as shown by the average trend line (Figure 3-2). As previously observed (Figure 3-1B), the wild type strain exhibited greater fold induction at early time points after exposure to inducing cues compared to the Δ*cpxP* mutant (Figure 3-2). In the double *cpxP degP* mutant, the fold induction of the pathway after addition of activating signal was comparable to the Δ*cpxP* mutant (Figure 3-2). Thus, CpxP is epistatic to DegP with respect to the Cpx signaling phenotypes. Further, both DegP and CpxP are required for wild type levels of Cpx pathway activation.

3.3.3 CpxP is an α-helical dimer that changes conformation, but is not misfolded, in the presence of the inducing cue, alkaline pH.

CpxP is a novel protein with no informative homologues. Thus, we have undertaken genetic analysis to identify important functional domains within CpxP (Buelow & Raivio, 2005). We also wished to characterize CpxP biochemically in order to learn more about its function. Consequently, we purified a MBP' (maltose binding protein)-'CpxP fusion protein (Raivio *et al.*, 1999), removed the N-terminal MBP moiety using an existing Factor Xa cleavage site, and subjected the resultant CpxP protein to circular dichroism (CD), size exclusion chromatography combined with multi-angle laser light scattering (MALLS), and small angle X-ray analysis (SAXS). In addition, we analyzed the oligomeric state of CpxP *in vivo* using formaldehyde cross-linking.

It is known that histidine kinases dimerize within the cell (Surette *et al.*, 1996). Thus, it seems possible that accessory proteins involved in signal transduction might also function as dimers. To test whether CpxP existed as a dimer, we undertook numerous approaches to analyze the oligomeric state of CpxP *in vivo* under physiological conditions and *in vitro* at pH 5.8 and 8.0. We over-expressed native CpxP or different CpxP fusion proteins *in vivo* and cross-linked with formaldehyde to determine the oligomeric state of CpxP. In the presence of no cross-linker a monomer of the expected molecular weight for MBP'-'CpxP,

CpxP'-'Bla and CpxP was observed (Figure 3-3 A,B,C, lane 1). However after formaldehyde was added, within 10 minutes of incubation, major cross-reactive protein species were present that exhibited double the weight of the respective monomer species, 114 kDa for MBP'-'CpxP, 98 kDa for CpxP'-'Bla and 34 kDa for CpxP (Figure 3-3 A, B, C, lane 2). This data suggests that *in vivo*, CpxP exists as a dimer.

To confirm this finding, we performed size exclusion chromatography in conjunction with MALLS to determine the oligomeric state of CpxP *in vitro* at pH 5.8 and 8.0. The elution volume of CpxP on the gel filtration column was independent of pH (Figure 3-4A). Light scattering data determined that at both pHs, CpxP formed a dimer (pH 5.8, MW = 33771 ± 1222 Da; pH 8.0, MW = 34360 ± 505 Da) (Figure 3-4A). In-line dynamic light scattering data allowed for an estimation of the apparent radius of hydration for the CpxP dimer particles. At pH 5.8, the R_H was 2.9 ± 0.4 nm, whereas at pH 8.0, the R_H was 3.0 ± 0.5 nm, indicating no change in the CpxP particle size within error. Thus, the light scattering data suggests that a shift to a more alkaline pH does not change the dimerization state of CpxP or size of the dimer particle.

SAXS is a useful technique for probing the structure and dynamics of macromolecules in solution. Scattering curves were collected at pH 5.8 and 8.0 to determine pH-dependent differences in CpxP structure at low resolution. The scattering curves deviate at $s > 0.19$ indicating the solution structures are not identical at low and high pH (Figure 3-4b). The particle distribution distance functions are similar, bell-shaped curves, indicative of a single compact, globular structure in both cases. Likewise, the Kratky plots at each pH, a plot of $I(s)s^2$ vs s, show both states are fully folded. The radius of gyration (*Rg*) and maximum particle dimension (D_{max}) calculated with GNOM were 22.9 and 66 Å for the low pH and 21.9 and 60 Å for the high pH state indicating the high pH CpxP state takes on a more compact structure than the low pH. Normalized standard deviations of the χ^2 values for the 15 independent GASBOR runs at pH 5.8 were 1.42 assuming a 149 amino acid monomer, 1.08 assuming a 298 amino acid dimer, and 1.11 for a 298 amino acid dimer restrained with P2 symmetry. At pH 8.0, the N.S.Ds were 1.36, 1.01 and 1.04, respectively. In both cases the assumption of a CpxP dimer in the GASBOR envelopes led to the lowest N.S.Ds, further evidence that CpxP is dimeric in solution.

Since the high resolution structure of CpxP is not available at the present time, we decided to perform circular dichroism studies to obtain information on the secondary and tertiary structure of the protein at pH 5.8 and 8.0. Far-UV experiments (255-190 nm) showed that wild type CpxP has a characteristic α-helical spectrum with two minima located at 208 and 222 nm (Figure 3-5 A). The spectrum did not change upon a shift to a higher pH indicating that both states are folded similarly. Using the DICHROWEB online server (Whitmore and Wallace, 2004) to deconvolute the spectra it was estimated that the percentage of α-helix is between 50-56% at pH 5.8 and 54-59% at pH 8.0. Near-UV experiments (255-320 nm) were used to further assess changes in the folding of CpxP at pH 5.8 and 8.0. In this range, CD signals for the individual aromatic amino acids can be seen at higher concentrations (1 mg/mL for this study) and the signals are highly dependent on the number of each residue and their chemical environments within the structure (Kelly *et. al.*, 2005). The spectrum of CpxP at each pH shows that it is folded into a stable structure with peaks corresponding to at least two of the three Phe residues (approximately 260 and 266 nm), the lone Tyr (approximately 278 nm) and the two Trp residues (maximum at 285 nm and minimum in pH 8.0 at ~297 nm) (Figure 3-5 B). The majority of peaks do not significantly change with a shift in pH with the exception of the minimum at 297 nm which is abolished at pH 5.8 and possibly the signal at 285 nm which may be decreased. The wavelengths of these peaks suggest they arise from tryptophan residues. This shows that small rearrangements may occur within CpxP tertiary structure in response to a pH shift from 5.8 to 8.0. To further test this, the CpxP CD at 220 nm (α-helix signal) was monitored as a function of temperature. The loss of CD signal at higher temperatures indicated a loss of helicity and conversion to a more unfolded state. The melting temperature (T_m) was measured for each pH and the results are summarized in Figure 3-5 C. At pH 8.0, the T_m of CpxP increases 3.3 °C showing that the protein adopts a slightly more stable structure. The near-UV CD experiments suggest that this conversion may be due to a slight rearrangement in the structure of CpxP to the more compact form demonstrated by SAXS. This may happen either in the core of CpxP or at the dimer interface.

3.3.4 CpxP LOF mutants exhibit proteolysis and dimerization phenotypes.

Previously, our lab isolated CpxP'-'Bla loss-of-function (LOF) mutants that no longer inhibited the Cpx pathway when over-expressed (Buelow & Raivio, 2005). These mutants fell into two classes, Class 1: those that had lost inhibitory ability but were otherwise present at comparable levels to the wild type CpxP'-'Bla protein, and Class 2: those that lost inhibitory ability and also had diminished protein levels relative to the wild type CpxP'-'Bla protein. We picked one representative from each class, CpxPD$_{61}$E'-'Bla for Class 1 and CpxPQ$_{55}$P'-'Bla for Class 2 and measured their half-lives. It was determined that the stability of CpxPD$_{61}$E'-'Bla was comparable to that of wild type with a half-life of 12 minutes and 30 secs ± 30 secs (Table 3-3, row 2). In contrast, the half-life of CpxPQ$_{55}$P'-'Bla was undetectable (Table 3-3, row 3). This data shows that, as expected, the Class 1 mutants exhibit stability comparable to that of the wild type, while the Class 2 mutants are grossly destabilized. We previously observed that the mutant LOF Class 2 CpxP'-'Bla proteins were detectable in a *degP* mutant (Buelow and Raivio, 2005). In order to determine if the Class 2 mutant defect was negated by restoration of protein levels, we examined Cpx pathway activity in wild type and *degP* null strains carrying a *cpxP'-lacZ*$^{+}$ transcriptional fusion. As expected, Class 1 LOF mutants remained unable to inhibit the Cpx response in either wild type or *degP* null strains (Figure 3-6, compare lanes 3 and 4 to lanes 5 and 6). Surprisingly, one Class 2 LOF mutant, CpxPQ$_{128}$H'-'Bla, regained the ability to inhibit the Cpx response in a *degP* mutant (Figure 3-6, compare lanes 3 and 4 to lanes 9 and 10). These observations demonstrate that, in all likelihood, the loss-of-function in the CpxPQ$_{128}$H'-'Bla is not due to gross misfolding of the protein, since Cpx pathway inhibition can be restored by eliminating DegP. Instead, it may be that the C-terminal region of CpxP is a target site(s) that is involved in recognition and/or degradation by the DegP protease.

To further characterize these LOF mutants and gain more insight into CpxP function, we tested whether the stable Class 1 mutants responded to inducing cues in a similar fashion to wild type CpxP'-'Bla. We grew strains expressing either wild type CpxP'-'Bla or one of the three Class 1 LOF mutants at pHs of 5.8 or 8.0 and compared protein levels. We were unable to detect any wild type CpxP'-'Bla protein at pH 8.0, as previously observed (Figure 3-7A, lanes 1 and 2)(Buelow & Raivio, 2005). Similarly, when we analyzed the Class 1 mutants we observed that the proteins were detectable at a pH 5.8 but not at pH 8.0 (Figure 3-7A, compare lanes 3, 5, and 7 to lanes 4, 6, and 8). Thus it seems that Class 1 CpxP'-'Bla mutants are degraded in the presence of Cpx inducing cues, in a similar fashion to the wild type CpxP'-'Bla protein.

We demonstrated that CpxP forms a dimer both *in vivo* and *in vitro* (Figures 4, 5). We were interested in determining if the loss of function phenotype displayed by our mutants, or the decreased stability we saw in the Class 2 LOF mutants (Buelow & Raivio, 2005) (Table 3-3) were the result of changes in the oligomeric state of the mutant proteins. Accordingly, we over-expressed native CpxP as well as a Class 1 mutant, CpxPD$_{61}$E, and a Class 2 mutant, CpxPQ$_{55}$P, in either a wild type or *degP* null background and cross-linked with formaldehyde to determine the oligomeric state of the proteins. In the presence of no cross-linker a monomer for all three proteins, CpxP, CpxPD$_{61}$E and CpxPQ$_{55}$P was observed (data not shown). However after the incubation with formaldehyde a major cross-reactive protein species was present that exhibited double the weight of the monomer, 34 kDa (data not shown). The relative amount of dimer to monomer varied between the three proteins. Thus, we quantified the bands and determined the ratio of dimer to monomer. The native CpxP dimer to monomer ratio was set at 1.0. We observed that the Class 2 LOF mutant, CpxPQ$_{55}$P, exhibited a similar dimer:monomer ratio to the native CpxP (Figure 3-7B, compare lanes 1 to 3). Surprisingly, the Class 1 LOF mutant, CpxPD$_{61}$E, had a dimer:monomer ratio that was three fold lower than wild type (Figure 3-7B, compare lanes 1 to 2). This data suggests that some Class 1 LOF mutants are defective in dimerization and this may influence their ability to inhibit the Cpx pathway. In contrast, Class 2 LOF mutations do not appear to affect the oligomeric state of CpxP.

3.4 Discussion.

In this study, we discovered new signal transduction phenotypes for CpxP and DegP by analyzing Cpx pathway activity immediately after the transition between inducing and non-inducing conditions. As hypothesized, CpxP is needed for the rapid shut-off of the pathway once envelope stress has been relieved (Figure 3-1A). Surprisingly, our results also demonstrated that both CpxP and DegP are needed for efficient induction of the Cpx response. Furthermore we demonstrated for the first time that DegP functions upstream of CpxP in the signaling pathway (Figure 3-2). In addition we showed that DegP mediated changes in CpxP stability differentially depending on the activating cue present (Table 3-3), suggesting that Cpx response induction can occur by at least two distinct mechanisms. To understand the role the novel CpxP protein plays in signal transduction in more depth we analyzed the structure of CpxP both *in vivo* and *in vitro*. We showed that CpxP forms an α-helical dimer and undergoes a conformational change in response to alkaline pH. Together, this data supports a model in which CpxP interacts with the CpxA sensor kinase dimer to fine-tune both induction and inhibition of the Cpx TCST system. In addition, our findings suggest that CpxP is targeted for proteolysis upon induction of the pathway by only a subset of inducers and that this involves a conformational change in CpxP, rather than complete misfolding. Analysis of LOF mutants argues that at least some of these may be defective in dimerization and that this is therefore a requirement for the role that CpxP plays in signaling.

3.4.1 Inducing cues differentially affect CpxP stability.

Previously it was shown that Cpx signal transduction was influenced by the proteolysis of CpxP (Buelow & Raivio, 2005, Isaac *et al.*, 2005). Steady state levels of CpxP were shown to diminish in the presence of alkaline pH and over-expression of PapG and PapE, but not when NlpE was over-expressed (Buelow and Raivio, 2005, Isaac *et al.*, 2005). We sought to quantify these differences by measuring half-lives of a CpxP'-'Bla protein in order to determine if this was a general response to inducing cues. The majority of inducing cues (pH, over-expression of BfpA, PapG and PapE) resulted in CpxP destabilization (Table 3-3) (Isaac *et al.*, 2005, Buelow & Raivio, 2005). However, upon deletion of DegP the stability of CpxP was returned under all these inducing conditions. Only one tested inducer, over-expression of NlpE, did not affect CpxP stability (Table 3-3) (Isaac *et al.*, 2005). Interestingly, the amino acid sequence of NlpE reveals a serine endoprotease inhibitor motif starting at position 99 (Snyder *et al.*, 1995). This suggests that one reason why CpxP was not degraded in response to NlpE over-expression may be that NlpE can inhibit DegP, thus preventing it from degrading CpxP. It has also been shown that NlpE, which is an outer membrane lipoprotein, acts as an accessory factor that is needed for the Cpx pathway to sense adhesion to abiotic surfaces (Otto & Silhavy, 2002). Thus, it may be that the adhesion signal is distinct from that generated by misfolded proteins, as our data and others have suggested (Table 3-3, DiGiuseppe *et al.* 2003, Isaac *et al.*, 2005).

3.4.2 New CpxP signaling phenotypes.

Our lab and others have characterized proteolysis of CpxP through over-expression of CpxP, either in its native form or as a fusion protein, and in a *cpxA** background, where the Cpx pathway is constitutively active (Buelow & Raivio, 2005, Isaac *et al.*, 2005, Raivio & Silhavy, 1997). In this study we analyzed signaling phenotypes in wild type and *cpxP* null strains immediately after the transition between inducing and non-inducing conditions, in order to identify phenotypes that may be more relevant physiologically. Surprisingly, it was observed that the wild type and *cpxP* mutant strains had different profiles for both activation and shut-off of the Cpx response (Figure 3-1). It has been shown that CpxP is important for the inhibition of the Cpx TCST pathway (Buelow & Raivio, 2005, Raivio *et al.*, 2000, Raivio *et al.*, 1999) and thus it was hypothesized that CpxP would play a role in inhibiting the pathway upon relief of envelope stress (Raivio *et al.*, 1999, Raivio *et al.*, 2000, Buelow & Raivio, 2005). This is what we observed. In a *cpxP* null strain it took the Cpx pathway 1.5 times longer to reach maximum shut-off compared to the wild type strain

(Figure 3-1A). This suggests that once the stress has been cleared from the periplasm, CpxP rapidly accumulates and is stabilized, allowing it to inhibit CpxA and return the pathway to a non-activated state in a timely fashion. Strikingly, we also found that CpxP was needed for proper induction of the Cpx response (Figure 3-1B). While eventually the wild type and *cpxP* mutant strains reached similar levels of induction, the initial rapid activation of the pathway required CpxP. This lag in initial activation of the Cpx response in the absence of CpxP could be because CpxA kinase activity is set at a higher threshold (Fleischer *et al.*, 2007) and therefore elevated levels of misfolded proteins are needed to activate the pathway relative to wild type. Thus, the presence of CpxP would protect the sensing domain of CpxA from signaling noise in the periplasm, allowing CpxA to be more sensitive when a threatening stress is present. It has been shown that BiP, an ER chaperone that binds to and inhibits Ire1 (Bertolotti *et al.*, 2000), a transmembrane kinase that senses and responds to ER stress (Cox *et al.*, 1993, Mori *et al.*, 1993), is not essential for Ire1 activity (Kimata *et al.*, 2004). It was suggested that BiP acts as an adjustor for Irel sensitivity to various ER stresses (Bertolotti *et al.*, 2000), which is what we are suggesting here for CpxP. There is also the possibility that CpxP itself acts as the initial trigger that activates the response. We showed that in response to alkaline pH, CpxP undergoes a conformational change that can be detected both in the overall shape of the CpxP dimer, as well as in the packing of hydrophobic residues as assessed by near-UV CD spectroscopy (Figure 3-4B). It has been hypothesized that CpxA undergoes a conformational change that would allow it to switch from a phosphatase to kinase. One could speculate that the conformational change observed in CpxP in the presence of inducing cues may enhance or facilitate the CpxA change in conformation favoring the kinase activity. Once the stress has been relieved CpxP would take on the low pH conformation, protecting the sensing domain of CpxA from false activation and promoting a conformational change in CpxA that inhibits the autokinase activity. Either way our data suggest that CpxP is acting as a gauge to sense how much stress is present in the envelope and to set the level of CpxA kinase activity accordingly. In the absence of CpxP, *E.coli* does not respond as efficiently to either the presence or absence of envelope stress.

Interestingly, we showed that DegP is needed for the proper activation of the Cpx pathway, because when it was absent the fold induction of the pathway never reached a level that was comparable to that of a wild type or a *cpxP* null strain (Figure 3-2). This agrees well with our previous observation that a CpxP'-'Bla fusion protein appears to accumulate to higher levels in a *degP* null strain (Buelow & Raivio, 2005). As a result, more inhibitor would be present and the Cpx pathway would be down-regulated. However, when we created a double knock out of *degP* and *cpxP*, the fold induction of the Cpx response was comparable to that of the *cpxP* null (Figure 3-2), showing that DegP functions upstream of CpxP in the signaling pathway. This is strong evidence that DegP is responsible for CpxP degradation under physiologically relevant conditions and that without proteolysis of CpxP, activation of the pathway is impeded. It was hypothesized that the activation of the Cpx pathway relied on the titration and/or degradation of CpxP to be activated (Raivio *et al.*, 2000). Our experiments confirm that the degradation of CpxP is important for the activation of the pathway (Figure 3-8). Degradation may involve prior binding of CpxP to misfolded proteins as suggested by Isaac *et al.* (2005).

3.4.3 Low resolution structure analysis of CpxP refines the model for its role in Cpx signal-transduction.

We hypothesized that an inducing cue needed to be present in order for DegP-mediated proteolysis of CpxP to occur, since over-expression of DegP alone did not result in increased pathway activity (Buelow & Raivio, 2005). It was speculated that the inducing cue either causes CpxP itself to become misfolded, which leads to its proteolysis, or that CpxP would interact with misfolded proteins and target them to DegP where they would jointly be degraded (Isaac *et al.*, 2005, Buelow & Raivio, 2005). Our results rule out the former model. SAXS and melting curve analyses showed that at high pH, a natural inducing cue for the Cpx response, CpxP does not unfold (Figures 3-4 and 3-5). Rather CpxP remains well folded and appears to undergo a conformational change, becoming more compact (Figures 3-4 and 3-5). Isaac *et al.* (2005) have shown that CpxP is required for the proteolytic degradation of misfolded P pilus subunits by DegP, suggesting that CpxP serves as an adaptor, interacting with and targeting misfolded proteins to the protease. Our SAXS analysis dos not reveal an obvious cleft or region where misfolded proteins might bind (Figure 3-4). However, these studies were performed on the purified protein *in vitro*, and thus we cannot rule out this model. The conformational change we observe at high pH is intriguing and may implicate a specific conformational switch that is important in DegP dependent proteolysis but this model awaits further experimentation.

3.4.4 CpxP is an α-helical dimer.

It has long been hypothesized that CpxP interacts with the sensing domain of CpxA to adjust its activity. Although no direct interaction has been shown, reconstitution studies showed that CpxP added to proteoliposomes containing only CpxA was sufficient to inhibit

Cpx pathway activity (Fleischer *et al.*, 2007). In addition, constitutively active *cpxA** alleles that contain mutations in the sensing domain are rendered signal blind and are not inhibited by CpxP over-expression, suggesting that an intact CpxA sensing domain is required to interact with CpxP (Raivio *et al.*, 1999). It is also known that HKs function as dimers (Surette *et al.*, 1996), as do RRs (Fiedler & Weiss, 1995, McCleary, 1996). Thus, it is not surprising that an accessory protein that facilitates CpxA signaling might also form a dimer. In this study we showed that CpxP forms an α-helical dimer *in vivo* under physiological conditions as well as *in vitro* at both pH 5.8 and 8.0. While this does not show a direct interaction between CpxA and CpxP, it does add another piece of evidence that supports an interaction between CpxP and CpxA. In addition, this data suggests that changes in oligomeric state are not associated with the roles that CpxP plays in fine tuning Cpx signaling.

3.4.5 CpxP LOF mutants alter dimerization and DegP-dependent proteolysis.

In this study we also further characterized several *cpxP* LOF mutations we previously isolated that alter either a highly conserved N-terminal domain or the C-terminus of CpxP and affect protein function (Class 1) or protein function and stability (Class 2). Surprisingly, the single Class 2 mutation, CpxPQ_{128}H, that falls in the C-terminus of the protein, seems to function solely by affecting DegP proteolysis. When this mutant (CpxPQ_{128}H) is placed in a *degP*$^-$ background, it regains the ability to inhibit the Cpx response (Figure 3-6). One possible explanation is that the C-terminus of CpxP contains a DegP proteolysis determinant. Interestingly, the near UV CD spectra of CpxP exhibit a maximum and a minimum at 285 nm and 297 nm, respectively, which correspond to the two Trp residues in CpxP (W30 and W149). The peak at 285 nm and the minimum at 297 nm show alterations between pH 5.8 and 8.0, suggesting a tertiary conformational change that affects these regions of the protein. Together, these data support the hypothesis that conformational changes that affect the C-terminus of CpxP in response to inducing cues may alter DegP dependent proteolysis. We also demonstrated that, although the Class 1 mutants are destabilized by Cpx pathway inducing cues, the same as the wild type protein (Figure 3-7A), some of these mutants are defective in dimerization (Figure 3-7B). We could detect formaldehyde cross-linked CpxPD_{61}E dimers at only about 30% the level of the wild type protein (Figure 3-7B). Thus, it is possible that dimerization is required for CpxP-mediated inhibition of the Cpx response and that some of our LOF mutants are defective because of an inability to dimerize as well as the wild type CpxP. Intriguingly, the amino acid change in the CpxPD_{61}E mutant is very conservative, which may suggest that it affects a highly specific protein:protein interaction. We also showed that the N-terminal Class 2 LOF mutant, CpxPQ_{55}P, formed dimers in vivo as well as its wild type counterpart (Figure 3-7B). Thus, the loss of stability observed for this mutant does not reflect a dimerization defect.

It is now clear that numerous TCST systems utilize accessory factors to expand their range of signal input or to fine tune signaling. Lipoproteins facilitate HK sensing of specific signals and in the absence of the lipoprotein the HK is blind to this signal (Majdalani *et al.*, 2005, Otto & Silhavy, 2002, Dartois *et al.*, 1997). It has been shown that NlpE, an outer membrane lipoprotein, acts as a positive accessory factor that is needed for the Cpx pathway to sense adhesion to abiotic surfaces (Otto & Silhavy, 2002). In addition, the *E. coli* RcsF and *Bacillus subtilis* KapB lipoproteins have been shown to be involved in the signal transduction of the Rcs and Kin pathways, respectively, in a similar fashion to NlpE. The Rcs pathway utilizes RcsF to help RcsC, the HK, sense specific conditions such as the presence of acidic phospholipids, excess Zn^{2+}, and low temperatures (Majdalani *et al.*, 2005). KapB is needed for the HK KinB to sense the trigger for sporulation (Dartois *et al.*, 1997). Lipoproteins can also negatively regulate TCST pathways. Such is the case with the lipoprotein, CseA, and the CseBC TCST system of *Streptomyces coelicolor* (Hutchings *et al.*, 2006). The CseBC system regulates the expression of σ^E activity in response to cell wall stress (Hutchings *et al.*, 2006). In addition to lipoproteins a new class of accessory factors appear to act as safeguards, protecting their respective pathways from unwarranted activation or signaling noise in the absence of genuine stress. They include CpxP and RseB of *E.coli*, TcpH of *Vibrio cholerae*, and BiP of the endoplasmic reticulum unfolded protein response. While all four of these proteins work as safeguards, they go about it in different ways. CpxP and BiP interact with their respective kinases, CpxA and IreI, altering their kinase activity (Bertolotti *et al.*, 2000, Kimata *et al.*, 2004, Raivio *et al.*, 2000, Raivio *et al.*, 1999, Fleischer *et al.*, 2007), while RseB and TcpH protect RseA, an anti-sigma factor that regulates the envelope stress responsive σ^E, and TcpP, a transcriptional regulator of the major virulence regulator *toxT*, respectively, from unwarranted proteolysis in the absence of a true signal (Cezairliyan & Sauer, 2007, Grigorova *et al.*, 2004, Ades *et al.*, 2003, Beck *et al.*, 2004, Matson & DiRita, 2005). Our studies show that the novel protein CpxP functions to set the sensitivity level of the Cpx response to misfolded proteins and to efficiently restore basal pathway activity upon stress relief. We present the first biochemical characterization of CpxP, which is implicated in the poorly understood processes of TCS sensing and the targeting of misfolded periplasmic proteins for proteolysis. We show that CpxP is a α-helical dimer that undergoes conformational changes in the presence of Cpx-activating signals that may be important for its *in vivo* roles (Figure 3-8).

Table 3-1. Bacterial strains used in chapter 3.

Strains	Description	Reference
MC4100	F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150* (Str^R) *relA1 flbB5301 decC1 ptsF25 rbsR*	(Casadaban, 1976)
TR50	MC4100 λRS88 (*cpxP'-lacZ*$^+$)	(Raivio & Silhavy, 1997)
TR61	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *ara*^{R}	This study
TR757	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *degP*::Tn10	(Buelow & Raivio, 2005)
TR932	TR50 (pPB)	(Buelow & Raivio, 2005)
TR1060	MC4100 (pJW15)	This study
TR1066	MC4100 (pJW25)	This study
DB4	TR757 (pPB)	(Buelow & Raivio, 2005)
DB10	TR50 (pPB1)	(Buelow & Raivio, 2005)
DB30	TR50 (pPB3)	(Buelow & Raivio, 2005)
DB31	TR50 (pPB2)	(Buelow & Raivio, 2005)
DB32	TR50 (pPB4)	(Buelow & Raivio, 2005)
DB84	TR61 (pNlpE-Kn)(pPB)	This study
DB85	TR61 (pBAD-Kn)(pPB)	This study
DB91	TR50 (pKDS301)(pPB)	This study
DB92	TR50 (ptrc99A)(pPB)	This study
DB96	TR757 (pKDS301)(pPB)	This study
DB97	TR757 (pKDS301)(pPB)	This study
DB223	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *cpxP::kan* (pMal2p)	This study
DB237	MC4100 λRS88 (*cpxP'-lacZ*$^+$)*malE*::Tn10	This study
DB239	DB237 (pCpxP)	This study
DB241	DB237 (pCpxPD$_{61}$E)	This study
DB260	MC4100 λRS88 (*cpxP'-lacZ*$^+$) Δ*degP*	This study
DB266	MC4100 *cpxP*::FRTKn	This study
DB271	MC4100 Δ*cpxP*	This study
DB275	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *malE*::Tn10 Δ*degP*	This study
DB283	DB275 (pCpxPQ$_{55}$P)	This study
DB298	DB271 (pJW15)	This study
DB299	DB271 (pJW25)	This study
DB300	MC4100 *degP*::Tn10	This study
DB304	DB300 (pJW15)	This study
DB305	DB300 (pJW25)	This study
DB329	MC4100 Δ*cpxP degP*::Tn10	This study
DB332	DB329 (pJW15)	This study
DB333	DB329 (pJW25)	This study
DB345	DB237 (pMCP)	This study
DB359	DB327 (pPB)	This study
SS1	TR757 (pMCP)	This study

Table 3-2. Plasmids used in chapter 3.

Plasmids	Descrpition	Reference
pBAD-Kn	Expression vector with an arabinose inducible promoter (Kn^R)	(Guzman *et al.*, 1995)
pCpxP	*cpxP* over-expression vector (Amp^R)	(Raivio *et al.*, 1999)
pCpxPD$_{61}$E	*cpxPD$_{61}$E* over-expression vector (Amp^R)	(Buelow & Raivio, 2005)
pCpxPQ$_{55}$P	*cpxPQ$_{55}$P* over-expression vector (Amp^R)	(Buelow & Raivio, 2005)
pCpxPU	*cpxP* gene and upstream flanking region cloned into pUC18 (Amp^R)	(Buelow & Raivio, 2005)
pCpxPKn	Kan cassette inserted into the BglII site of pCpxPU	This study
pJW15	promoterless *luxCDABE* reporter construct (Kn^R)	(MacRitchie *et al.*, 2007)
pJW25	*cpxP* promoter cloned into pJW15 (Kn^R)	(MacRitchie *et al.*, 2007)
pKDS301	p*trc*99A bases *bfpA* over-expression vector (Amp^R)	(Stone *et al.*, 1996)
pMal2p	Cloning vector that is used to make periplasmic MBP fusion proteins (Amp^R)	New England Biolabs
pMAK705	A cloning vector that uses a temperature-sensitive pSC101 replicon for allelic exchange (Cam^R)	(Hamilton *et al.*, 1989)
pMAK705-CpxPKn	pMAK705 containing *cpxP::kan* from pCpxPKn (Cam^R, Kn^R)	This study
pMCP	Over-expresses a functional MBP-CpxP fusion protein (Amp^R)	(Raivio *et al.*, 1999)
pND18	*nlpE* gene cloned into pBAD18 (Amp^R)	(Danese *et al.*, 1995)
pNlpE-Kn	0.9kb *Eco*RI-*Xmn*I fragment from pND18 cloned into pBAD-Kn (Kn^R)	This study
pPB	CpxP’-‘Bla translational fusion over-expression vector (Amp^R, Cam^R)	(Buelow & Raivio, 2005)
pPB1	pPB encoding a CpxPD$_{61}$E’-‘Bla mutation (Amp^R, Cam^R)	(Buelow & Raivio, 2005)
pPB2	pPB encoding a CpxPQ$_{55}$P’-‘Bla mutation (Amp^R, Cam^R)	(Buelow & Raivio, 2005)
pPB3	pPB encoding a CpxPR$_{60}$Q’-‘Bla mutation (Amp^R, Cam^R)	(Buelow & Raivio, 2005)
pPB4	pPB encoding a CpxPD$_{61}$V’-‘Bla mutation (Amp^R, Cam^R)	(Buelow & Raivio, 2005)
p*trc*99A	High expression vector with a multiple cloning site following an IPTG inducible *trc* promoter (Amp^R)	Pharmacia
pUC18	Cloning vector (Amp^R)	Invitrogen
pUC4K	Cloning vector (Kn^R)	Invitrogen

Table 3-3. Half-life analysis of CpxP'-'Bla in response to Cpx inducing cues.[a]

Protein being Detected[b]	Condition[c]	Half-life value (min:sec)[d]	Strain name
1 CpxP'-'Bla	uninduced	10:14 ± 2:28	TR932
2 CpxPD$_{61}$E'-'Bla	uninduced	12:30 ± 0:30	DB10
3 CpxPQ$_{55}$P'-'Bla	uninduced	undetectable	DB31
4 CpxP'-'Bla	pH 5.8	>30	TR932
5 CpxP'-'Bla	pH 7.8	undetectable	TR932
6 CpxP'-'Bla	pH 8	undetectable	TR932
7 CpxP'-'Bla	*degP* null	>30	DB4
8 CpxP'-'Bla	*degP* null ; pH 7.8	>30	DB4
9 CpxP'-'Bla	*degP* null ; pH 8	>30	DB4
10 CpxP'-'Bla	pBAD vector control for NlpE	9:08 ± 0:09	DB85
11 CpxP'-'Bla	over-expression of NlpE	7:37 ± 0:03	DB84
12 CpxP'-'Bla	p*trc*99A vector control for BfpA	8:30 ± 0:32	DB92
13 CpxP'-'Bla	over-expression of BfpA	undetectable	DB91
14 CpxP'-'Bla	*degP* null, p*trc*99A	>30	DB97
15 CpxP'-'Bla	*degP* null, over-expression BfpA	>30	DB96
16 Bla	wild type	>30	DB92
17 BAP	wild type	>30	TR932

a. Half lives of CpxP'-'Bla were measured after the addition of 0.5mg/mL of spectinomycin

b. β-lactamase is abbreviated Bla, Bacterial alkaline phosphatase is abbreviated BAP, Maltose binding protein is abbreviated MBP.

c. Overnight cultures were subcultured 1:50 and induced as indicated for about 4 hours. Induction by pH was done using 100mM Na-phosphate at various pH's. Over-expression of NlpE and BfpA was accomplished using 0.2% arabinose and 1mM IPTG, respectively.

d. Protein density was quantified and graphed versus time. The graph was used to determine the protein half-life. Ranges in half-life values are represented using ±. Each half-life was determined a minimum of two times.

Half-life experiments performed by Daelynn R. Buelow

A)

Strain	Time to maximum shut off (mins)	Fold shut off
Wild type	100 ± 8.66	3.82 ± 0.89
cpxP null	150 ± 0	4.76 ± 2.10

B)

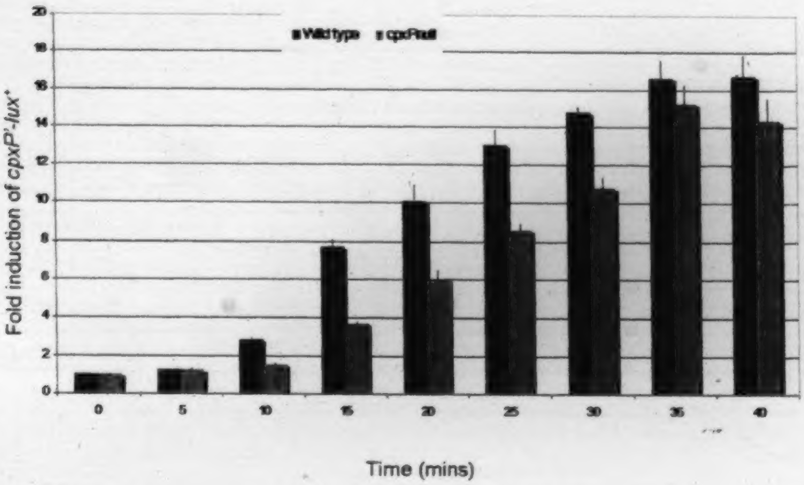


Figure3-1. CpxP is involved in the activation and inhibition of the Cpx pathway. A) Inhibition of the Cpx pathway after envelope stress takes longer in a *cpxP* mutant. After a downshift from pH 8.0 to 5.8, Cpx pathway activity was measured using a *cpxP'-lux*$^+$ reporter. Pathway activity was measured every 5 minutes for the first hour and then every 15 minutes until a plateau was reached to determine time to maximum shut off. Fold shut off was determined by comparing maximum activation to maximum inhibition for each strain. Luciferase activity from the vector control strains was negligible (TR1060 and DB298). Standard deviations for time to maximum shut off and fold shut off are indicated. B) Activation of the Cpx pathway is less efficient in a *cpxP* mutant. Luciferase produced from a plasmid carrying a *cpxP'-lux*$^+$ fusion was measured as a reporter of Cpx-mediated gene expression in response to pH induction in a wild type (TR1066) and *cpxP* null (DB299) background. Light production was measured immediately after shift to pH 8.0 from pH 5.8 and then measured in 5 minute intervals for 40 minutes. Induction levels were determined by comparing pathway activity at different time points to time zero for each strain. Error bars represent standard deviation. Activation and shut-off experiments performed by Daelynn R. Buelow

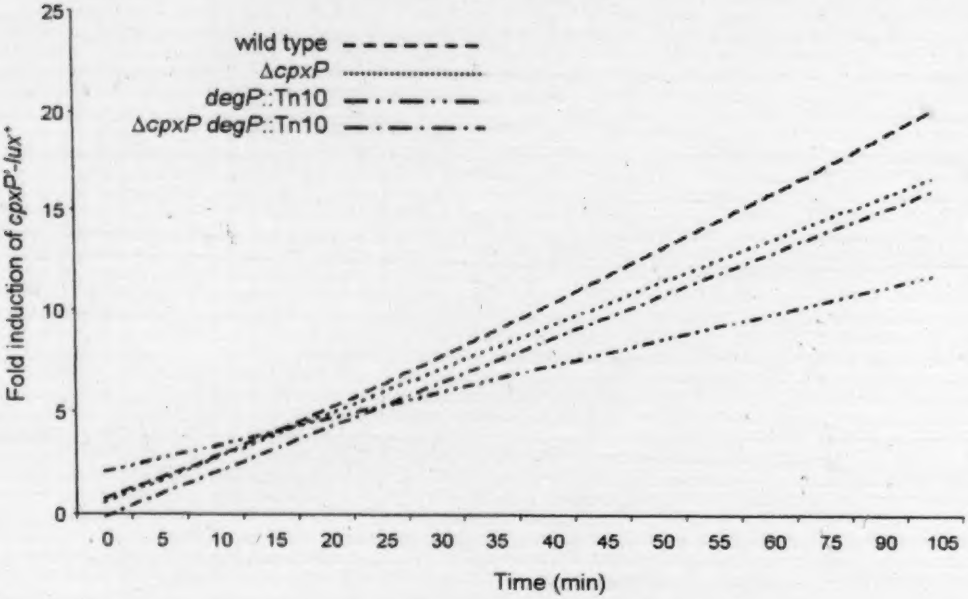


Figure 3-2. CpxP and DegP influence Cpx pathway activity and DegP functions upstream of CpxP. Activation of the Cpx pathway in wild type (TR1066), Δ*cpxP* (DB299), *degP*::Tn10 (DB305), and Δ*cpxP degP*::Tn10 (DB333) backgrounds after an upshift from pH 5.8 to 8.0. Cpx pathway activity was measured using luciferase produced from a plasmid carrying a *cpxP'-lux*$^+$ fusion. Cpx-mediated gene expression was measured in triplicate for each time point. Fold induction was determined by comparing a given time point to time zero for each strain and the average trend line for induction is shown. Luciferase vector controls (TR1066, DB298, DB304, and DB332) for each strain had negligible values. Activation experiment performed by Daelynn R. Buelow

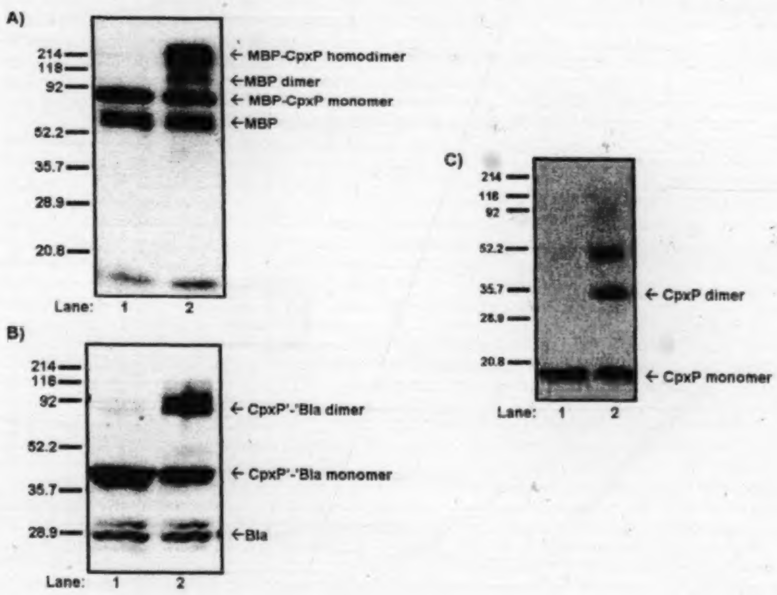

A)
214
118
92
52.2
35.7
28.9
20.8
← MBP-CpxP homodimer
←MBP dimer
← MBP-CpxP monomer
←MBP
Lane: 1 2
B)
214
118
92
52.2
35.7
28.9
← CpxP'-'Bla dimer
← CpxP'-'Bla monomer
←Bla
Lane: 1 2
C)
214
118
92
52.2
35.7
28.9
20.8
← CpxP dimer
← CpxP monomer
Lane: 1 2

Figure 3-3. CpxP forms a dimer *in vivo*. Western blots were performed on whole-cell lysates of wild type strains expressing different CpxP fusion proteins that had been cross linked with 1% v/v formaldehyde for 10 minutes. A) Cross-linking of MBP-CpxP (DB345) B) Cross-linking of CpxP'-'Bla (DB359) C) Cross-linking of native CpxP (DB239) expressed from the p*trc* promoter. Lane 1 represents uncross-linked sample, while lane 2 represents cross-linked sample after 10 minutes in the presence of formaldehyde. Each blot was performed three times and a representative blot is shown. Cross-linking experiments performed by Daelynn R. Buelow.

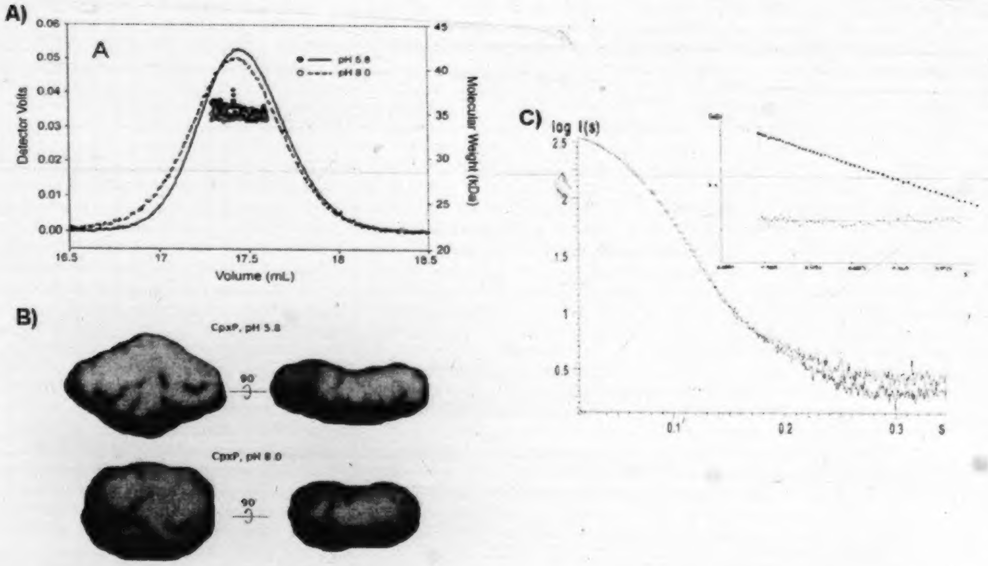
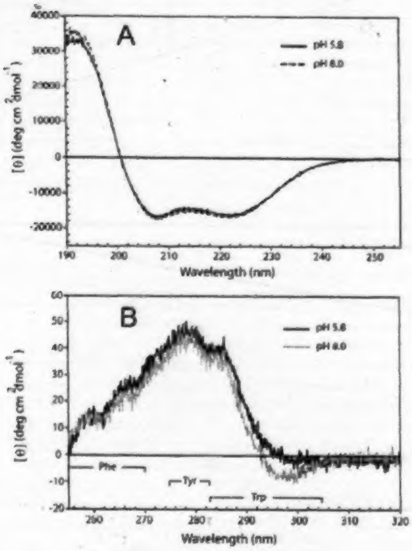


Figure 3-4. CpxP is a dimer at pH 5.8 and 8.0 but has slightly different conformations. A) Size-exclusion chromatography and multi-angle laser light scattering determination of molecular weight of CpxP at pH 5.8 and 8.0. Light scattering over the elution profile of CpxP shown in solid (pH 5.8) and dashed (pH 8.0) lines. Superimposed across the peaks in filled (pH 5.8) and empty (pH 8.0) circles indicate molecular weights as a function of elution volume. B) Small-angle X-ray scattering curves for wildtype CpxP in 250 mM NaCl, 50 mM sodium phosphate, 1mM EDTA, 10% glycerol, pH 5.8 (blue curve) and pH 8.0 (red curve). Inset, the Guinier Plot for the scattering at pH 5.8 for *s.Rg* < 1.3 fit by a straight line indicating absence of aggregation in the sample (similarly for pH 8.0, data not shown). C) *Ab initio* low-resolution solution envelopes for wildtype CpxP at pH 5.8 (top) and 8.0 (bottom) calculated using GASBOR. Each envelope is an average of 15 GASBOR runs assuming a dimer of 298 amino acids with P2 symmetry enforced. Characterization of CpxP dimer *in vitro* done by David C. Arthur and Ross A. Edwards.



C

pH	Melting Temperature (°C)
5.8	48.3 ± 0.2
8.0	51.6 ± 0.2

Figure 3-5. CpxP is mostly α-helical and slightly changes conformation but is not misfolded in response to the inducing cue of alkaline pH. A) Far-UV spectrum of CpxP in 50 mM sodium phosphate pH 5.8 (solid) and 8.0 (dashed). B) Near-UV spectrum of CpxP in 250 mM NaCl, 1 mM EDTA, and 50 mM sodium phosphate pH 5.8 (black) or 8.0 (grey). The wavelength ranges for the signals of Phe, Tyr and Trp are indicated at the bottom. C) Summary of the melting temperatures for CpxP at pH 5.8 and 8.0. Samples were in 250 mM NaCl, 1 mM EDTA and 50 mM sodium phosphate pH 5.8 or 8.0. Each of the CD experiments was repeated in duplicate and representative spectra are shown. Characterization of the secondary and tertiary structure of CpxP *in vitro* done by David C. Arthur.

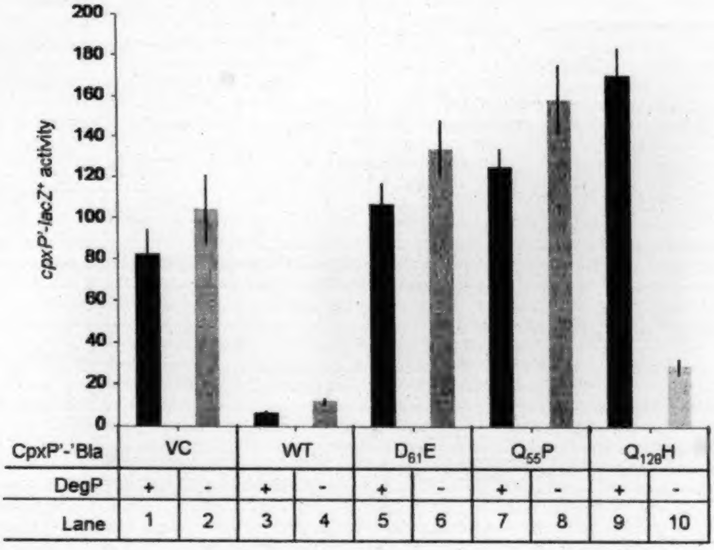


Figure 3-6. Inhibitory activity can be returned to the C-terminal Class 2 LOF CpxP mutant, CpxPQ$_{128}$H'-'Bla in a *degP* mutant background. A chromosomal *cpxP'-lacZ*$^+$ fusion was used to determine the effect of overproduction of CpxP'-'Bla fusion proteins had on the Cpx pathway in a *degP* null background. β-galactosidase levels were measured from wild type (odd lanes) and *degP* null strains (even lanes) carrying wild type and mutant CpxP'-'Bla over-expression plasmids. Strains used in this experiment are as follows TR930 (lane 1), DB5 (lane 2), TR932 (lane 3), DB4 (lane 4), DB10 (lane 5), DB70 (lane 6), DB30 (lane 7), DB71 (lane 8), DB40 (lane 9), and DB75 (lane 10). VC refers to vector control, WT stands for wild type. Characterization of CpxP LOF mutants in the present and absent of *degP* was done by Daelynn R. Buelow.

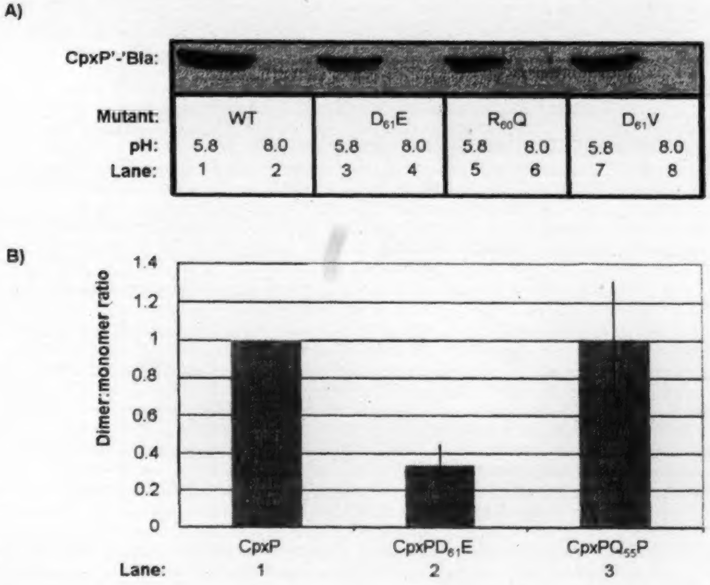


Figure 3-7. Characterization of CpxP LOF mutants. A) Class 1 LOF CpxP'-'Bla mutant protein levels are diminished in a similar fashion to wild type CpxP'-'Bla in response to inducing cues. Western blots were carried out on whole-cell lysates of each strain carrying the CpxP'-'Bla encoding plasmid, pPB (TR932) (lanes 1 and 2) or the mutated pPB plasmids, CpxPD$_{61}$E'-'Bla (DB10) (lanes 3 and 4), CpxPR$_{60}$Q'-'Bla (DB30) (lanes 5 and 6) and CpxPD$_{61}$V'-'Bla (DB32) (lanes 7 and 8) that had been grown in LB buffered with sodium phosphate buffer at a pH 5.8 (lanes 1, 3, 5, and 7) or pH 8.0 (lanes 2, 4, 6, and 8). B) Class 1 LOF CpxP'-'Bla mutants are defective at dimerization. Western blots were preformed on whole cell lysates that were cross linked with 1% v/v formaldehyde for 15 minutes. To determine the ratio of dimer to monomer, the density of the monomer and dimer bands were determined and the dimer density was divided by the monomer density. Ratio of dimer to monomer was graphed for wild type (DB239)(lane 1), CpxPD$_{61}$E (DB241)(lane 2), and CpxPQ$_{55}$P (DB283)(lane 3). Further characterization of CpxP LOF mutants was carried out by Daelynn R. Buelow.

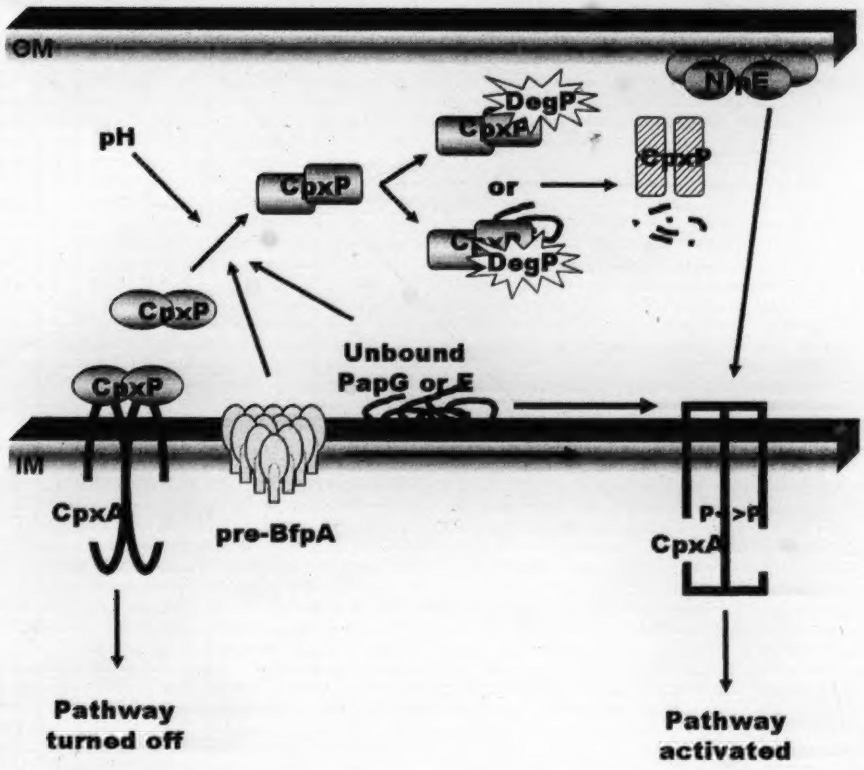

OM
pH
CpxP
DegP
CpxP
or
CpxP
CpxP
DegP
CpxP
CpxP
Unbound
PapG or E
IM
CpxA
pre-BfpA
P->P
CpxA
NlpE
Pathway
turned off
Pathway
activated

Figure 3-8. Model for activation of the Cpx pathway. Under nonstressful conditions CpxP (light oval form) is a dimer that interacts with the sensing domain of CpxA (central region of the periplasmic domain of CpxA), maintaining the pathway in an off state. In response to stresses such as pH, over-expression of pre-BfpA or over-expression of unbound PapG or E, CpxP undergoes a conformational change (square form), which may be needed to prime CpxA for activation. This conformational change also may be needed for DegP and/or misfolded protein recognition and subsequent degradation. Not all inducing cues of the Cpx pathway result in the degradation of CpxP. When NlpE (light ovals associated with the outer membrane) is over-expressed there is activation of the pathway, which does not involve the degradation of CpxP, suggesting that there are at least two mechanisms for Cpx pathway activation. OM = outer membrane, IM = inner membrane, P = phosphate.

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Chapter 4:

The highly conserved, uncharacterized transcription factor YifE and other cytoplasmic factors influence Cpx regulon expression independently of CpxRA*

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* a version of this chapter has been submitted to Microbiology

4.1 Introduction.

In their natural environment, bacteria encounter threatening changes that can be potentially lethal. Bacteria must be able to respond and adapt to these hostile environments in order to survive. One way this is done is through stress responses, which can be compartmentalized into cytoplasmic or extracytoplasmic responses. The extracytoplasmic stress response of *Escherichia coli* allows the bacteria to monitor the envelope, which consists of the outer membrane, inner membrane, and periplasm. To date the extracytoplasmic stress response of *E.coli* is controlled by at least 6 regulatory pathways; the σ^E, Cpx (conjugal pilus expression), Bae (bacterial adaptive response), Psp (phage shock protein), Rcs (regulator of capsular synthesis) and the outer membrane vesicle release responses.

The Cpx pathway is regulated by a two component regulatory system that consists of an inner membrane bound histidine kinase, CpxA, a cytoplasmic response regulator, CpxR and a periplasmic accessory factor, CpxP (Dong *et al.*, 1993, Weber & Silverman, 1988, Danese & Silhavy, 1998). It has been shown that the Cpx pathway is activated in the presence of alkaline pH (Danese & Silhavy, 1998, Nakayama & Watanabe, 1995), over-expression of an outer membrane lipoprotein, NlpE (Snyder *et al.*, 1995), over-expression of the enteropathogenic *E.coli* type IV bundle-forming pilus subunit, BfpA (Nevesinjac & Raivio, 2005), over-expression of the uropathogenic *E.coli* P pilus subunits, PapG or PapE, in the absence of their periplasmic chaperone, PapD (Jones *et al.*, 1997) and accumulation of enterobacterial common antigen intermediate lipid II in the inner membrane (Danese *et al.*, 1998). Based on these inducing cues, and the genes that comprise the Cpx regulon, it is thought that the Cpx pathway is involved in monitoring the presence of misfolded and mislocalized proteins within the periplasm, more specifically those that are associated with the periplasmic face of the inner membrane.

Under non-stressful conditions, CpxP is hypothesized to interact with the periplasmic sensing domain of CpxA and maintain the pathway in an off or down regulated state. In the presence of inducing cues, CpxP changes conformation, possibly to prime CpxA to respond to the stresses present and/or to make itself a more suitable substrate for DegP, a periplasmic serine endoprotease (Buelow *et al.*, 2007). Subsequently it is hypothesized that CpxP is titrated away from CpxA either alone or in conjunction with misfolded proteins and is degraded by DegP (Buelow et al., 2007, Buelow & Raivio, 2005, Isaac *et al.*, 2005). This leaves the sensing domain of CpxA free to perceive the stresses present and autophosphorylate itself on a conserved histidine residue (Raivio & Silhavy, 1997). The phosphate is subsequently transferred to CpxR on a conserved aspartate residue (Raivio & Silhavy, 1997). When CpxR is phosphorylated its ability to bind to its consensus sequence is enhanced and consequently it increases transcription of the Cpx regulon (Raivio & Silhavy, 1997, Pogliano *et al.*, 1997, Danese & Silhavy, 1997). Within the Cpx regulon there are numerous protein folding and degrading factors such as DegP, PpiA, a peptidyl-prolyl-isomerase (Liu & Walsh, 1990, Pogliano et al., 1997), and DsbA, the major periplasmic disulfide oxidase (Bardwell *et al.*, 1993, Danese & Silhavy, 1997, Kamitani *et al.*, 1992, Pogliano et al., 1997). Along with the increased transcription of these protein folding and degrading factors Cpx response activation also increases transcription of the genes encoding CpxRA, CpxP and Spy, a periplasmic protein of unknown function (Raffa & Raivio, 2002, Danese & Silhavy, 1998, Raivio *et al.*, 2000, Raivio *et al.*, 1999). Once the stress has been cleared from the envelope, CpxP is hypothesized to return to its initial conformation and interact with the sensing domain of CpxA, shutting down the response and preventing false activation (Buelow et al., 2007).

Although the Cpx pathway is activated by unique activating cues and upregulates genes specific to the Cpx pathway, there is some overlap with other envelope stress responses. It has been shown that the Cpx, Bae and σ^E pathways are all activated when PapG is over-expressed in the absence of PapD (Jones et al., 1997, Raffa & Raivio, 2002). Also, in response to stressful environments the Cpx and σ^E pathways both up-regulate *degP* expression while the Bae and Cpx pathways up-regulate *spy* expression (Raffa & Raivio, 2002, Connolly *et al.*, 1997, Danese *et al.*, 1995). Further, the Cpx response is proposed to be involved in the regulation of both the σ^E response and the cytoplasmic heat shock response (De Wulf *et al.*, 2002). These observations suggest a level of integration between stress responses, which may aid the bacteria in adapting to stressful environments more rapidly or prepare the bacteria for any additional stresses it may encounter.

Genes that are regulated by extracytoplasmic stress responses are normally thought to be required for adaptation to envelope stresses. However, recently it was shown that σ^E pathway activation during stationary phase is independent of extracytoplasmic signal transduction (Costanzo & Ades, 2006). Costanzo and Ades (2006) demonstrated that this activation was due to ppGpp, suggesting that the σ^E pathway is activated in response to both external and internal environments. It was also shown that the Cpx pathway is activated in a growth dependent manner independently of CpxA (DiGiuseppe & Silhavy, 2003). Further, Button *et al.* (2007) showed that mutation of *ydcQ*, which encodes a putative DNA binding protein and is a suppressor of σ^E lethality, caused down-regulation of numerous envelope stress responses. These observations suggest that both internal and external environments regulate the Cpx pathway.

Before the identification of DegP as the protease that degrades CpxP (Buelow & Raivio, 2005, Isaac et al., 2005), we initiated a screen to find factors that, when mutated, caused a decrease in Cpx expression. Although we set out to identify a potential protease that degraded CpxP, our screen identified a DNA-dependent transcription regulator, YifE, which affects the regulation of the Cpx regulon independently of CpxRA. We observed that YifE is a global regulator that may respond to cytoplasmic misfolded proteins. Finally, we showed that additional cytoplasmic regulatory inputs influence the Cpx regulon, including ppGpp and the family of DnaJ-like chaperones.

4.2 Materials and Methods.

4.2.1 Bacterial strains.

Bacterial strains used in this study are listed in Table 4-1. All strains were constructed using standard genetic techniques (Silhavy *et al.*, 1984). Primers used for polymerase chain reaction (PCR) and sequencing are listed in Table 4-2.

4.2.2 Media, antibiotics and growth conditions.

All strains were grown on Luria-Bertani (LB) agar (Silhavy et al., 1984) at 37°C. Initial screening for regulators of *cpxP* expression was on lactose-MacConkey agar (Difco), which is an indicator media that measures β-galactosidase activity. Liquid cultures were grown in LB broth with aeration at 37°C. Strains were maintained with the appropriate selection,100 μg/mL ampicillin (amp), 25 μg/mL chloramphenicol (cam), 30 μg/mL kanamycin (kan), 30 μg/mL spectinomycin (spc), or 25 μg/mL tetracycline (tet). All antibiotics were purchased from Sigma.

4.2.3 Transposon mutagenesis screen for regulators of *cpxP* expression.

To identify genes that regulated *cpxP* expression, a strain (DB8) carrying a plasmid that over-expressed CpxP and a *cpxP'-lacZ*$^+$ fusion integrated on the chromosome in single copy on the λRS88 phage was used. This strain was subjected to transposon mutagenesis via P1 transduction using lysate pools grown on Tn*10-cam* mutagenized MC4100. MC4100 was mutagenized using the mini-Tn*10* delivery vehicle λNK1324 as previously described by Kleckner *et al.* (1991). Mutagenized DB8 were plated onto LB amp cam plates and grown overnight. The next day colonies were patched onto lactose-MacConkey plates containing the same antibiotics and grown for a minimum of 24 hours. Since we set out to identify the protease that degrades CpxP, mutants with a strong Lac$^-$ phenotype were chosen for further analysis.

4.2.4 Sequencing of the *yifE::*Tn10-*cam* and λ*plac*Mu53 insertion points.

Determining the insertion points of the Tn*10-cam* in *yifE* and the randomly inserted λ*plac*Mu53 elements was done using arbitrarily-primed PCR as described previously by Caetano-Annoles (1993). Briefly, a single colony containing the insertion of interest was used in a low stringency PCR reaction with the out1-L (Tn10-*cam*) or Mu3'2 (λ*plac*Mu53) and ARB1 primers (Table 4-2). out1-L is a primer that is complementary to the Tn*10-cam* element. Mu3'2 is a primer that is complementary to the λ*plac*Mu53 element. The product of the low stringency PCR was cleaned using a QIAquick PCR purification kit (QIAGEN), according to the manufacturer's directions and used as the substrate for a second high stringency PCR reaction. The high stringency PCR reaction used the 1-L primer (Tn10-*cam*) or the Mu3'1 primer (λ*plac*Mu53) (Table 4-2). The 1-L primer is complementary to the IS10 sequence contained at the ends of the Tn*10-cam* element and the Mu3'1 primer is complementary to the Mu termini of the Mu53 element. The ARB2 primer (Table 4-2), binds to the end of the amplified product resulting from the PCR with the ARB1 primer. The high stringency PCR product was cleaned as previously described for the low stringency PCR product and used as a template for sequencing using the protocol for the DYEnamic ET terminator cycle sequencing premix kit (Amersham) provided by the Molecular Biology Service Unit (University of Alberta) and the 1-L or Mu3'1 primer. Sequence data obtained from the arbitrarily-primed PCR product was BLAST searched using BacMap (Stothard *et al.*, 2005) and the *Escherichia coli* K12 MG1655 sequence.

4.2.5 Construction of *relA*::kan, *dnaK*::kan, *dksA*::kan and *yifE*::kan mutants.

Strains harboring the *relA*::kan, *dnaK*::kan, *dksA*::kan or *yifE*::kan alleles were constructed by transduction using P1 lystates that had been grown on the corresponding Keio library strain (Baba *et al.*, 2006).

4.2.6 β-galactosidase assay.

Single colonies of each bacterial strain to be assayed were inoculated into 2mL LB broth with the appropriate antibiotics and grown for 14 to 16 hours at 37°C with aeration. Those carrying derivatives of the pCA plasmids were induced with 0.1mM IPTG (Invitrogen). The next day, β-galactosidase activity was measured using the microtiter plate assay (Slauch & Silhavy, 1991), with slight variation to the protocol; all assays were carried out on overnight cultures unless otherwise stated. For those assays carried out on 6 hour cultures, overnight cultures were subcultured 1:50 into 2 mL fresh medium with appropriate antibiotics and grown at 37°C. 1M sodium phosphate buffer (Sambrook *et al.*, 1989) at the appropriate pH was sometimes added to the subcultures to a final concentration of 100mM to induce the Cpx response. Additionally, 5 μL of cell lysate was added to 195 μL of Z-buffer due to high expression of β-galactosidase in the overnight cultures. The β -galactosidase measurements were then multiplied by 10 to account for the dilution. Each assay was performed in triplicate. Error bars represent the standard deviation.

4.2.7 λp*lac*Mu53 infection.

Infection of MC4100 by λp*lac*Mu53 was performed according to Bremer *et al.* (1985). Briefly, 1mL of MC4100 grown overnight was mixed with 10^8 λp*lac*Mu53 phage and 10^9 λp*lac*Mu507 helper phage. The mixture was incubated for 30 minutes at room temperature, 5mL of LB containing 10mM sodium citrate was added to the mixture and the cells were pelleted. The pellet was resuspended in 1mL LB and a 10^{-2} dilution was plated onto LB + kan plates and grown overnight at 37°C.

4.3 Results.

4.3.1 A screen for factors affecting cpxP'-lacZ$^+$ expression.

Initially, we were interested in identifying factors that influenced CpxP inhibition of the Cpx response. As a result we initiated a screen that could identify proteases that affected CpxP stability, or other factors that, when knocked out, increased the inhibition CpxP exerts on the pathway. For this screen we used a strain that over-expressed CpxP from an exogenous promoter and carried a *cpxP'-lacZ*$^+$ transcriptional fusion. This strain was subjected to transposon mutagenesis with a mini-Tn10 derivative that encodes chloramphenicol resistance (Tn10-*cam*)(Kleckner et al., 1991). We screened for a phenotype of white or Lac$^-$ colonies on lactose-MacConkey indicator media after a minimum of 24 hours of growth hoping to identify genes that when mutated, stabilized CpxP and therefore enhanced down-regulation of the Cpx response. One transposon insertion showed a dramatic decrease in *cpxP'-lacZ*$^+$ expression upon isolation and retransduction into the reporter strain. Although this insertion conferred a decrease in pathway activity it did not result in an increase in CpxP stability (data not shown). Upon sequence and BLAST search analysis it was determined that the Tn10-*cam* resided in the *yifE* gene (Figure 4-1A).

The databases indicate that YifE is a highly conserved cytoplasmic protein. The exact function of YifE is not known but it is predicted to be a putative DNA binding protein involved in regulation of transcription. While YifE was not a factor that affected CpxP stability, we decided to further characterize it because of its marked effects on *cpxP* expression.

4.3.2 *yifE*::Tn10-*cam* is a gain-of-function mutation.

The Tn10-*cam* transposon that we used can create either loss-of-function or gain-of-function mutations (Pratt & Silhavy, 1996, Raffa & Raivio, 2002, Kleckner et al., 1991). Consequently, we first determined whether the *yifE*::Tn10-*cam* allele exhibited a gain-of-function or a loss-of-function phenotype. To determine what effect the *yifE*::Tn10-*cam* allele had on Cpx-regulated gene expression we used a *cpxP'-lacZ*$^+$ fusion in single copy on the chromosome and compared the β-galactosidase activity of a strain bearing the *yifE*::Tn10-*cam* allele to that of a strain carrying a plasmid that over-expressed YifE (pCA-YifE)(Kitagawa *et al.*, 2005) and a strain that contained a *yifE* deletion marked with a kanamycin resistance cassette (Baba et al., 2006). When the *yifE*::Tn10-*cam* allele was present there was a four to six fold decrease in *cpxP'-lacZ*$^+$ expression (Figure 4-1B, compare lanes 1 and 2). In strains over-expressing YifE there was a six to ten fold decrease in pathway activity compared to wild type (Figure 4-1B, compare lanes 3 and 4). In contrast, the *yifE*::kan deletion allele had no effect on Cpx-mediated gene expression (Figure 4-1B, compare lanes 5 and 6). Thus, we conclude that the *yifE*::Tn10-*cam* allele is a gain-of-function mutation.

4.3.3 YifE and the CpxRA two component system function independently to control expression of Cpx-regulated genes.

It was previously observed that the Cpx pathway underwent a growth dependent activation in stationary phase that is independent of CpxA (DiGiuseppe & Silhavy, 2003). In addition, mutations in the uncharacterized gene *ydcQ* that suppress lethality due to loss of σ^E also lead to down-regulated expression of Cpx-controlled genes independently of the CpxRA signal transduction pathway (Button et al., 2007). This observation suggests that there are Cpx-independent regulators that influence expression of the Cpx regulon. To investigate the mechanism by which the *yifE*::Tn10-*cam* allele affected *cpxP* expression, we examined expression of additional Cpx-regulated genes and performed epistasis tests with a *cpxR*::spc insertion allele. We compared expression of the Cpx-regulated gene *dsbA* during log and stationary phase growth as measured from a *dsbA'-lacZ*$^+$ reporter construct in a wild type background or in backgrounds that contained either or both the *cpxR*::spc and *yifE*::Tn10-*cam* alleles (Figure 4-2A). As previously observed, when CpxR was absent there is a decrease in *dsbA* expression (Figure 4-2A, compare lanes 1 and 5 to lanes 3 and 7, respectively). In addition, when the *yifE*::Tn10-*cam* allele was present there was a decrease in *dsbA* expression (Figure 4-2A, compare lanes 1 and 5 to lanes 2 and 6, respectively). Thus, the *yifE*::Tn10-*cam* allele affects multiple Cpx-regulated genes. Interestingly, when both the *cpxR*::spc and *yifE*::Tn10-*cam* alleles were present there was an additional decrease in expression of *dsbA* (Figure 4-2A, compare lanes 4 and 8 to all other lanes). Similar results were obtained when YifE was over-expressed in wild type and *cpxR*::spc backgrounds, as well as when *dsbA'-lacZ*$^+$ expression was examined in wild type, *cpxA* null, *yifE*::Tn10-*cam* and *cpxA*$^-$ *yifE*::Tn10-*cam* double mutants (data not shown). We performed the same experiments using *cpxP'-'lacZ*$^+$ and *degP'-lacZ*$^+$ reporter constructs and observed the same trends (data not shown), which suggests that YifE works independently of the CpxRA two component system to down-regulate expression of multiple Cpx-regulated genes.

The above observations suggest that YifE works independently of the CpxRA pathway to influence expression of Cpx-regulated genes. To confirm the independence of the YifE and CpxRA regulators we expressed CpxR from an exogenous promoter (pUC19-CpxR) in a *yifE*::Tn10-*cam* background and measured expression from either a *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ transcriptional fusion. The levels of β-galactosidase produced were then compared to a wild type strain carrying the same transcriptional fusions. It was observed that the strains bearing the *yifE*::Tn10-*cam* allele and carrying the pUC19-CpxR vector had similar or higher gene expression levels to the wild type strain (Figure 4-2B, compare lanes 3 and 6 to lanes 1 and 4, respectively). Since over-expression of CpxR normally leads to a large increase in expression of the *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ reporters (data not shown), this result again indicated that the effects of YifE and CpxR are additive, and therefore they function in separate pathways.

Lastly, we ruled out any influence of YifE on Cpx signal transduction by comparing the ability of wild type and *yifE*::Tn10-*cam* mutants to induce the Cpx response. We grew wild type and *yifE*::Tn10-*cam* strains bearing either a *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ reporter construct at pH 5.8, which has been shown to repress Cpx pathway activity or pH 8.0, where the Cpx pathway is induced (Danese & Silhavy, 1998) and measured β-galactosidase activity. β-galactosidase activity at pH 8.0 was compared to that at pH 5.8 for each strain to determine the fold induction of the Cpx response. As observed previously (Danese & Silhavy, 1998), wild type strains were induced between two and four fold by alkaline pH, depending on the reporter construct used (Figure 4-2C, lanes 1 and 3). Similarly, strains bearing the *yifE*::Tn10-*cam* allele were also induced two to four fold depending on the reporter construct used (Figure 4-2C, lanes 2 and 4). Thus, the ability to induce the Cpx pathway in a *yifE*::Tn10-*cam* strain is not compromised, implying that YifE does not affect Cpx signal transduction.

4.3.4 YifE regulates other envelope stress responses and is a global regulator of gene expression.

Given that the *yifE*::Tn10-*cam* allele affects the regulation of Cpx regulon members and that some of Cpx regulon members such as *spy* and *degP* are co-regulated by other envelope stress responses, we wanted to determine if the *yifE*::Tn10-*cam* allele also affected the regulation of genes controlled by other envelope stress responses. We looked to see if the *yifE*::Tn10-*cam* allele had any effect on genes regulated by the σ^E and the BaeSR envelope stress responses. To measure expression of σ^E regulated genes, we used a *rpoHP3'-lacZ*$^+$ reporter fusion in single copy on the chromosome. When *rpoHP3* expression was compared between wild type and *yifE*::Tn10-*cam* strains, we observed an 11 fold decrease (Figure 4-3A, lanes 1 and 2). We also measured expression of a Bae-regulated gene using a *mdtA'-lacZ*$^+$ fusion derived from a chromosomal insertion of λ*plac*Mu53 into the *mdtA* gene. Similar to what we observed with *rpoHP3* expression, the *yifE*::Tn10-*cam* allele decreased the expression of *mdtA* compared to wild type strains. However, in this case, there was only a two fold decrease (data not shown). Thus, it appears that the *yifE*::Tn10-*cam* allele affects the regulation of multiple envelope stress responses.

Since the *yifE*::Tn10-*cam* allele affects genes belonging to the σ^E, Cpx and Bae envelope stress responses, we wanted to determine if the *yifE*::Tn10-*cam* allele was a pleiotropic regulator of gene expression. To do this we utilized a library of mutants that carried a promoterless *lacZ* gene on a hybrid lambda phage (λ*plac*Mu53) randomly inserted into the chromosome. We selected 48 random mutants and measured β-galactosidase expression in the presence and absence of the *yifE*::Tn10-*cam* allele. β-galactosidase levels were elevated in the presence of the *yifE*::Tn10-*cam* allele in 13 of these strains and down-regulated in 17 (data not shown). 18 strains were unaffected by the *yifE*::Tn10-*cam* allele (data not shown). We sequenced the region of the genome flanking seven of the positively regulated insertions and seven of the negatively regulated insertions (Table 4-3). The sequencing data revealed that the *yifE*::Tn10-*cam* allele affects expression of a wide variety of genes with functions that include transport, metabolism and energy production (Table 4-3). Since expression of 30 of 48 randomly selected *lac* reporters was affected by the *yifE*::Tn10-*cam* allele, this data suggests that YifE exerts global effects on gene expression.

4.3.5 Expression of Cpx regulon genes is affected by other cytoplasmic regulators in addition to YifE.

It has been shown mutants of DjlA, a member of the DnaJ chaperone family (Genevaux *et al.*, 2001a, Genevaux *et al.*, 2001b), causes an increase in Rcs envelope stress response pathway activity (Shiba *et al.*, 2006) . The Rcs pathway senses disruptions in the peptidoglycan layer and induces the formation of capsule (Huang *et al.*, 2006, Majdalani &

Gottesman, 2005). As well it has been shown that ppGpp is needed for the activation of genes regulated by the σ^E pathway in stationary phase in a manner that is independent of RseA, the anti-sigma factor (Costanzo & Ades, 2006). Thus, we tested whether DjlA or its homologues DnaJ and CbpA, as well as ppGpp, had any affects on the expression of Cpx regulon members. To do this we measured β-galactosidase activity from either a *cpxP'-'lacZ*$^+$ or *degP'-lacZ*$^+$ transcriptional fusion in single copy on the chromosome in the presence and absence of mutations that eliminated *djlA, dnaJ, cbpA,* or ppGpp. When a *djlA::*spc allele was introduced into strains carrying a *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ reporter, there was a dramatic decrease in β-galactosidase activity (Figure 4-4A, compare lanes 1 and 5 to lanes 4 and 8, respectively). Likewise, in a *dnaJ*::Tn10-42*tet* mutant there was also a dramatic decrease in expression of *cpxP* and *degP* (Figure 4-4A, compare lanes 1 and 5 to lanes 3 and 7, respectively). In contrast, in a *cbpA*::kan mutant there was no effect on expression of either *cpxP* or *degP* (data not shown). When mutations affecting the components involved in ppGpp formation, *relA* and *dksA*, were introduced into the reporter strains, there was also a decrease in expression of *cpxP* (Figure 4-4C, compare lanes 1 to lanes 3 and 4). Thus, it seems that the expression of Cpx-regulated genes is influenced by numerous cytoplasmic factors.

Since RelA, DksA, DnaJ and DjlA all seem to influence expression of the Cpx regulon, we wanted to determine if any of them functioned in the same pathway as YifE. Due to the dramatic decrease in gene expression seen in the *djlA*::*spc* mutant it was hard to determine the epistasis of DjlA with respect to YifE (Figure 4-4A). However, epistasis experiments employing the *dnaJ*::Tn10-42*tet* and *yifE*::Tn10-*cam* alleles revealed that the double mutant exerted an additive effect on expression of *cpxP* (Figure 4-4B). When the *relA::kan yifE*::Tn10-*cam* and *dksA::kan yifE*::Tn10-*cam* double mutants were examined, we observed an intermediate phenotype relative to the individual mutations (Figure 4-4C, comparing lanes 1, 2, 3, and 5 to each other and lanes 1, 2, 4, and 6 to each other). Thus, no epistatic relationship exists between *yifE*::Tn10-*cam* and any of the *dnaJ, dksA* or *relA* mutant alleles. These observations suggest that these factors are working independently of each other.

4.3.6 DnaK is epistatic to YifE.

Given that both DnaJ and DjlA affect the regulation of Cpx controlled genes, we wanted to test if their co-chaperone DnaK (Genevaux et al., 2001b, Liberek *et al.*, 1991) had a similar effect. To determine the effect DnaK had on expression of genes controlled by the Cpx response, we measured β-galactosidase activity produced from *degP'-lacZ*$^+$ and *dsbA'-lacZ*$^+$ reporter constructs. In a *dnaK::kan* background there was a 15 fold decrease in *degP* expression and a 4 fold decrease in *dsbA* expression relative to the wild type background (Figure 4-5A and 4-5B, compare lanes 1 and 3). Since DnaK had an effect on expression of the Cpx-regulated genes *dsbA* and *degP*, we assayed epistasis of the *yifE*::Tn10-*cam* and *dnaK::kan* alleles. We found that the double *yifE*::Tn10-*cam dnaK::kan* mutant had similar levels of β-galactosidase activity to the *yifE*::Tn10-*cam* single mutant when measuring *degP* expression (Figure 4-5A, compare lanes 2, 3, and 4). However when comparing the expression of *dsbA* in the double *yifE*::Tn10-*cam dnaK::kan* mutant to the individual *yifE*::Tn10-*cam* and *dnaK::kan* mutants the β-galactosidase activity of the double mutant was in between that of the individual mutants (Figure 4-5B, lanes 2, 3 and 4). Thus, it seems that the *yifE*::Tn10-*cam* allele is epistatic to DnaK with respect to *degP* expression.

4.4 Discussion.

In this study, we took advantage of a mini-Tn10 element to identify factors that affected the expression of the Cpx regulon. We identified a transposon insertion, *yifE*::Tn10-*cam*, that caused a decrease in expression of genes regulated by the Cpx response (Figure 4-1B, 4-2). We demonstrated that *yifE*::Tn10-*cam* is a gain-of-function mutation (Figure 4-1B) that affects Cpx regulated gene expression independently of signal transduction mediated by the CpxRA two component system (Figure 4-2 and 4-3). Additionally, we showed that YifE appears to exert global effects on gene expression, and these include an alteration in the expression of genes regulated by the σ^E and Bae envelope stress responses (Table 4-3, Figure 4-3).

4.4.1 YifE influences expression of envelope stress genes.

We showed that in addition to the *yifE*::Tn10-*cam* allele affecting the expression of Cpx regulon members *dsbA*, *degP*, and *cpxP*, that it also affects the expression of other envelope stress response genes, such as *rpoH* and *mdtABCD*, which belong to the σ^E and Bae stress responses, respectively (Figures 4-2 and 4-3). Despite the fact that YifE negatively regulates expression of the Cpx regulon, it does this independently of the CpxRA two component signal transduction system (Figure 4-2). Further, in the presence of alkaline stress, the Cpx pathway is still induced the same fold in the presence of the *yifE*::Tn10-*cam* allele as in a wild type strain (Figure 4-2C). Thus, the effects of the *yifE*::Tn10-*cam* allele are completely independent from the CpxRA signaling pathway. We can therefore conclude that the decrease in Cpx-regulated gene expression caused by this allele is not due to a reduction of envelope stress that is sensed and transduced by the CpxA sensor kinase and CpxR response regulator. As activation of the Cpx response is not impeded by the gain-of-function *yifE*::Tn10-*cam* allele (Figure 4-2), this suggests that the sensing of envelope stress is a priority for the cell and that in the presence of envelope stress CpxR~P is able to over-ride other negative regulators of the Cpx regulon to elicit an adaptive response. Although we did not determine the epistasis of YifE with respect to the σ^E and BaeSR regulators, based on our observations with the Cpx response, we think it unlikely that YifE is down-regulating expression of *rpoHP3* and *mdtABCD* by decreasing envelope stress sensed by σ^E and BaeSR, respectively.

4.4.2 A function for the highly conserved, uncharacterized protein, YifE.

YifE is a highly conserved, uncharacterized protein, which is predicted to be a transcriptional regulator. We showed that the *yifE*::Tn10-*cam* allele exerts global effects on gene expression (Table 4-3, Figures 4-2 and 4-4). In addition, we demonstrated that YifE negatively regulates envelope stress responses and factors involved in transport and metabolism, while positively regulating at least two putative oxidoreductases, *yghZ* and *ydgJ*, a peptidase, and genes involved in energy production and conservation (Table 4-3). Thus, YifE appears to affect the expression of a large number of genes that encode proteins in all cellular compartments and encompass a broad range of functions. The effects of YifE may be greater during stationary phase than log phase since the *yifE*::Tn10-*cam* allele exerted a larger effect on gene expression at this stage of growth (Figure 4-2 and data not shown). Interestingly, the *yifE* knockout has no effect on gene expression but the over-expression of YifE mimics that of the *yifE*::Tn10-*cam* allele (Figure 4-1B). Thus, it seems that YifE is likely involved in a stress response since clearly YifE does not affect basal levels of gene expression. The mini Tn10 element inserted into YifE between codon 87 and 88, thus affecting the last 25 amino acids of the protein (Figure 4-1A). Given that the *yifE*::Tn10-*cam* allele is a gain-of-function mutation that mimics over-expression of the wild type allele, we hypothesize that the C-terminal region of YifE is important in governing its activity, while the N-terminal region is involved in gene regulation. Thus the protein produced from the *yifE*::Tn10-*cam* allele is still able to bind DNA and cause altered gene expression, but it now does this in an uncontrolled manner.

Intriguingly, we showed that repression of the Cpx regulated gene *degP* in a *yifE*::Tn10-*cam dnaK::kan* double mutant was similar to that of a *yifE*::Tn10-*cam* single mutant, suggesting that YifE functions downstream of DnaK. DnaK is a molecular chaperone that has been shown to be involved in the heat shock response (Bukau & Horwich, 1998), where it helps promote either the refolding or degradation of misfolded proteins. Thus, it appears that the regulatory activities of YifE may be linked to cytoplasmic misfolded proteins. It has been shown that DnaK acts as an anti-sigma factor for σ^{32} (σ^{H}) under normal conditions (Nakahigashi *et al.*, 1995, Tomoyasu *et al.*, 1998, Nagai *et al.*, 1994, McCarty *et al.*, 1996). However, under heat shock conditions, it is thought that DnaK is titrated away by misfolded proteins in the cytoplasm, allowing σ^{32} to interact with RNAP (Yura & Nakahigashi, 1999). Perhaps DnaK negatively regulates YifE in a similar fashion. In the presence of a stressor, such as cytoplasmic misfolded proteins, DnaK would be sequestered away to help alleviate the stress, relieving the inhibition of YifE, which would then go on to inhibit and activate the expression of genes that would facilitate adaptation (Figure 4-6). Clearly, as *yifE* is not essential, its role must be in fine-tuning the adaptive response.

Interestingly, this relationship between DnaK and YifE was seen with respect to *degP* expression (Figure 4-5A), which has been shown to be regulated by both the σ^{E} and Cpx pathways. However, with *dsbA* expression the epistasis between DnaK and YifE was not as straightforward. The *yifE*::Tn10-*cam dnaK::kan* double mutant had intermediate levels of *dsbA'-lacZ*$^{+}$ expression compared to the single mutants (Figure 4-5B). Thus, we cannot conclude if YifE is downstream of DnaK with respect to *dsbA* regulation. The ambiguous results seen in the DnaK YifE epistasis with *dsbA* expression might be due to additional regulators that have yet to be identified.

4.4.3 Numerous cytoplasmic signals influence regulation of genes controlled by the Cpx response.

Costanzo and Ades (2006) showed that σ^{E} activity was increased in stationary phase in an RseA independent manner. The Cpx response is also induced in stationary phase, independently of the CpxA sensor kinase (DiGiuseppe & Silhavy, 2003). The increase in σ^{E} activity is due to the accumulation of ppGpp (Costanzo & Ades, 2006). We were curious to see if ppGpp accumulation affected regulation of genes controlled by the Cpx response.

We observed a decrease in expression of Cpx regulon genes when ppGpp production was eliminated (Figure 4-4C). Interestingly, ppGpp has been shown to be involved in the regulation of other alternative sigma factors, σ^{54} and σ^{S}, upon entry into stationary phase (Jishage *et al.*, 2002, Laurie *et al.*, 2003). Thus, ppGpp is proposed to be involved in a coordinate response to entry into stationary phase. It is known that bacteria experience numerous physiological changes upon entry into stationary phase (Nystrom, 2004) and ppGpp may be needed to coordinate these physiological changes. Our results indicate that regulation of envelope stress genes may be a part of the adaptation mediated by ppGpp (Figure 4-4C).

DjlA, which acts as a co-chaperone with DnaK, has also been shown to increase the Rcs envelope stress response when mutated (Shiba et al., 2006). Thus, we also examined the effects of mutations in DjlA, along with its homologues, DnaJ and CbpA (Genevaux et al., 2001a, Genevaux et al., 2001b) on expression of Cpx regulon genes (Figure 4-4A and 4-4B). We showed that elimination of DnaJ or DjlA also down-regulated the expression of Cpx regulated genes. Although the mechanism for how these chaperones affect gene expression is not known, this, in conjunction with regulation by ppGpp and YifE shows there are many ways of coordinating the expression of envelope stress response genes with situations that occur in the cytoplasm. In agreement with this, Button *et al.* (2007) showed that a putative DNA-binding protein, YdcQ, also influences gene expression of Cpx regulon members independently of CpxRA. While stress responses are described as cytoplasmic and extracytoplasmic, here we show, along with others, that there is communication between these responses. This might allow for the activation of the responses together to prime the bacteria in response to dramatic physiological changes, as may be the case with ppGpp. Alternatively, the role of this communication in some situations may be to down regulate one response so that another can deal with more pressing stresses, as we suggest might be the case with YifE.

Table4-1. Strains and plasmids used in chapter 4.

Strain or plasmids	Description	Reference
Strains		
MC4100	F$^-$ *araD139 Δ(argF-lac)U169 rpsL150* (Str^R) *relA1 flb5301 decC1 ptsF25 rbsR*	(Casadaban, 1976)
TR49	MC4100 λRS88 (*degP'-lacZ*$^+$)	(Raivio & Silhavy, 1997)
TR50	MC4100 λRS88 (*cpxP'-lacZ*$^+$)	(Raivio & Silhavy, 1997)
TR71	MC4100 λRS88 (*rpoHP3'-lacZ*$^+$)	This study
TR96	MC4100 λRS88 (dsbA'-*lacZ*$^+$)	(Raffa & Raivio, 2002)
GP110	MC4100 *dnaJ*::Tn*10-42* Δ*djlA*::*Ωspc*	(Genevaux et al., 2001a)
DB19	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *yifE*::Tn*10-cam*	This study
DB20	MC4100 λRS88 (*degP'-lacZ*$^+$) *yifE*::Tn*10-cam*	This study
DB23	MC4100 λRS88 (*dsbA'-lacZ*$^+$) *yifE*::Tn*10-cam*	This study
DB26	MC4100 λRS88 (*dsbA'-lacZ*$^+$) *cpxR::spc*	This study
DB28	MC4100 λRS88 (*dsbA'-lacZ*$^+$) *yifE*::Tn*10-cam cpxR::spc*	This study
DB137	MC4100 λRS88 (*cpxP'-lacZ*$^+$) Δ*djlA*::*Ωspc*	This study
DB138	MC4100 λRS88 (*degP'-lacZ*$^+$) Δ*djlA*::*Ωspc*	This study
DB141	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *dnaJ*::Tn*10-42tet*	This study
DB142	MC4100 λRS88 (*degP'-lacZ*$^+$) *dnaJ*::Tn*10-42tet*	This study
DB162	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *yifE*::Tn*10-cam dnaJ*::Tn*10-42tet*	This study
DB170	DB19 (pUC19)	This study
DB171	DB20 (pUC19)	This study
DB174	DB19 (pUC19-CpxR)	This study
DB175	DB20 (pUC19-CpxR)	This study
DB203	MC4100 λRS88 (*rpoHP3'-lacZ*$^+$) *yifE*::Tn*10-cam*	This study
DB288	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *relA::kan*	This study
DB289	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *dksA::kan*	This study
DB292	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *yifE*::Tn*10-cam relA::kan*	This study
DB293	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *yifE*::Tn*10-cam dksA::kan*	This study
DB352	MC4100 λRS88 (*degP'-lacZ*$^+$) *dnaK::kan*	This study
DB353	MC4100 λRS88 (*dsbA'-lacZ*$^+$) *dnaK::kan*	This study
DB354	MC4100 λRS88 (*degP'-lacZ*$^+$) *yifE*::Tn10-*cam dnaK::kan*	This study
DB355	MC4100 λRS88 (*dsbA'-lacZ*$^+$) *yifE*::Tn*10-cam dnaK::kan*	This study
DB366	TR50 (pCA-YifE)	This study
DB367	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *yifE*::kan	This study
Plasmids		
pCA-YifE	YifE over-expression vector	(Kitagawa et al., 2005)
pUC19	Cloning vector (Amp^R)	Invitrogen
pUC19-CpxR	*cpxR* gene was amplified using *finpho* and CpxR3'*Eco* and cloned into pUC19 BamH1 and EcoR1 sites (Amp^R)	This study

Table 4-2. Primers used for PCR in chapter 4.

Primer	Sequence 5'-3'
1-L	CTG CCT CCA GAG CCT G
ARB 1	GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN GAT AT
ARB 2	GGC CAC GCG TCG ACT AGT AC
CpxR3'*Eco*	CGG AAT TCC GGT TAA GCT GCC TAT CAT
finpho	ACA TTA ACA GGA GGC TGT TCG TGC
Mu3'1	AAA TTT GCA CTA CAG GCT TGC
Mu3'2	CGG CAT AAG CTG ATT TGT GA
out1-L	CAG GCT CTC CCC GTG GAG

Table4-3. Random λ*plac*Mu53 insertions and envelope stress genes that exhibit regulation by *yifE*.[a]

Gene name	Alternative name	Proposed operon structure	Function[c]
Negatively regulated[b]			
ddlA		*ddlA*	-D-ala D-ala ligase
glgP	*glgY*	*glgP*	-glycogen phosphorylase; carbohydrate transport/metabolism
hemX		*hemC hemD hemX hemY*	-uroporphyrinogen III methyltransferase; coenzyme transport/metabolism
kptA	*yjiI*	*b4331 kptA*	-RNA 2' phosphotransferase; translation, ribosome structure and biogenesis
xdhC	*ygeC*	*xdhA ygeT xdhC*	-xanthine dehydrogenase; energy production/conversion
ycfJ		*ycfJ*	-hypothetical periplasmic protein
ygiE		*ygiE*	-zinc and other divalent cation uptake transporter; inorganic transport/metabolism
cpxP	*finE, yiiO*	*cpxP*	-periplasmic accessory protein involved in activation and repression of the Cpx response
degP	*htrA, ptd*	*degP*	-periplasmic serine endoprotease
dsbA	*dsf, iarA, ppfA*	*yihE dsbA*	-periplasmic disulfide oxidase
rpoH	*fam, hin, htpR*	*rpoH*	-heat shock sigma factor
mdtA	*yegM*	*mdtA mdtB mdtC mdtD baeS baeR*	-multidrug efflux pump
Postively regulated[b]			
abgB	*ydaI*	*abgA abgB abgT ogt*	-peptidase; required for p-aminobenzyl-glutamate utilization
hybA	hybL	*hybO hybA hybB hybC hybD hybE hybF hybG*	-hydrogenase 2 component; energy production/conversion
uvrD	*dar2, dda, mutU, pdeB, rad, recL, srjC, uvr502, uvrE*	*uvrD*	-ATP dependent 3'-5' DNA helicase II
ybhC		*ybhC*	-acyl-Co thioesterase; lipoprotein
ydgJ		*ydgJ*	-oxidoreductase
ygbN		*ygbN*	-gluconate permease
yghZ	*mgrA*	*yghZ*	-oxidoreductase; energy production/conversion

a. β-galactosidase activity was measured in 48 random strains bearing promoterless λplacMu53 insertions in the presence and absence of the *yifE*::Tn*10cam* allele. The identity of some of the genes that contained λ*plac*Mu53 insertions that yielded altered levels of β-galactosidase in the presence of the *yifE*::Tn10-*cam* allele is indicated

b. $P<0.05$

c. Functions listed as described on Ecogene (http://ecogene.org/index.php) and BacMap (http://wishart.biology.ualberta.ca/BacMap/index.html)

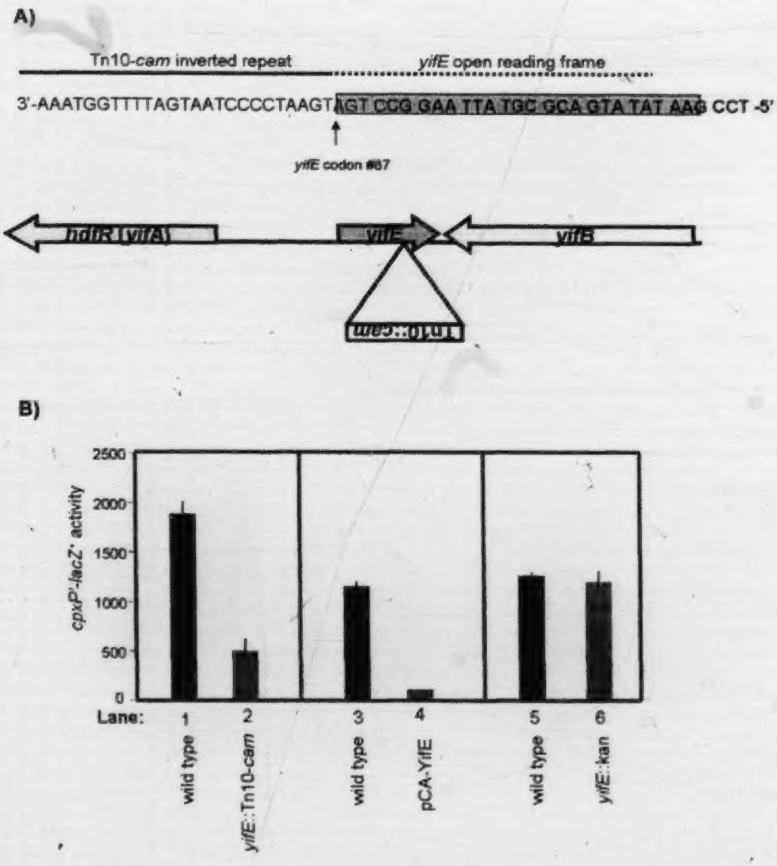

Figure 4-1. *yifE*::Tn10-*cam* is a gain of function mutation. A) The mini Tn10-*cam* element that causes decreased *cpxP'-lacZ*$^+$ expression resides in the *yifE* gene. The insertion point of the mini Tn10-*cam* element is represented diagrammatically below, while the exact nucleotide sequence of the junction between the mini Tn10-*cam* and *yifE* is shown above. B) β-galactosidase produced from a *cpxP'-lacZ*$^+$ transcriptional fusion was measured after growth to late stationary phase in wild type (TR50)(lanes 1, 3, and 5), *yifE*::Tn10-*cam* (DB19) (lane 2), and *yifE*::kan (DB367)(lane 6) backgrounds, as well as in a transformant bearing the ASKA over-expression vector pCA-YifE (DB366)(lane 4). Each assay was performed in triplicate and the mean and standard deviation are presented.

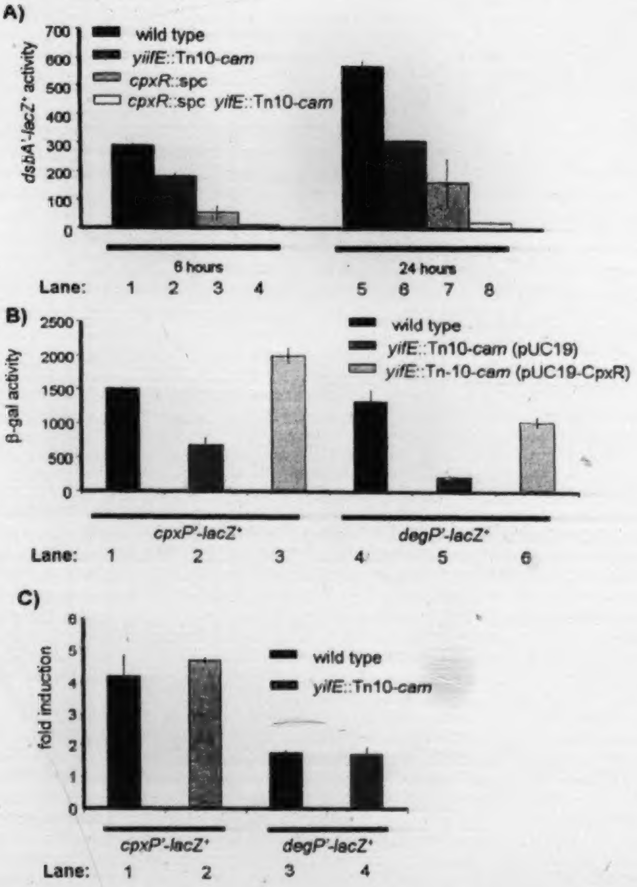

Figure 4-2. YifE regulates Cpx regulon genes independently of CpxRA. A)β-galactosidase levels were measured to determine the epistasis of the *yifE*::Tn10-*cam* and *cpxR::spc* alleles. Cpx-regulated gene expression was measured using a *dsbA'-lacZ*$^+$ fusion at 6 hours and 24 hours in wild type (TR96)(lanes 1 and 5), *yifE*::Tn10-*cam* (DB23)(lanes 2 and 5), *cpxR*::spc (DB26)(lanes 3 and 7), and *cpxR*::spc *yifE*::Tn10-*cam* double mutants (DB28)(lanes 4 and 8) backgrounds. B) The effects of *yifE*:Tn10-*cam* and CpxR overexpression are additive. β-galactosidase activity was measured either from a *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ transcriptional fusion after overnight growth in wild type (lanes 1 and 4), and *yifE*::Tn10-*cam* backgrounds (lanes 2,3,5, and 6) carrying pUC19 (vector control)(lanes 2 and 5) or pUC19-CpxR (lanes 3 and 6). The strains assayed were TR50 (lane 1), DB170 (lane 2), DB174 (lane 3), TR49 (lane 4), DB171 (lane 5), DB175 (lane 6). C) The Cpx response is activated normally in strains carrying a *yifE*::Tn10-*cam* allele. β-galactosidase levels were measured from either a *cpxP'-'lacZ*$^+$ or a *degP'-lacZ*$^+$ fusion in mid log phase (OD_{600}=0.6) in wild type or *yifE*::Tn10-*cam* strains that had either been grown at a pH of 5.8 or 8.0. The fold induction was determined by comparing the β-galactosidase activity of the strains grown at pH 8.0 to that of the strains grown at pH 5.8. The strains assayed were TR50 (lane 1), DB19 (lane 2), TR49 (lane 3), DB20 (lane 4). Experiments were performed in triplicate and the means and standard deviations are presented.

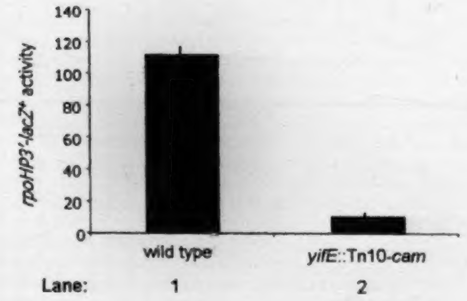

Figure 4-3. YifE affects the regulation of the σ^E-regulated *rpoHP3* promoter. β-galactosidase levels were measured from an *rpoHP3'-lacZ*$^+$ reporter fusion after 24 hours of growth in wild type (TR71) (lane 1) and *yifE*::Tn10-*cam* (DB203) (lane 2) backgrounds. Assays were performed in triplicate and the means and standard deviations are presented.

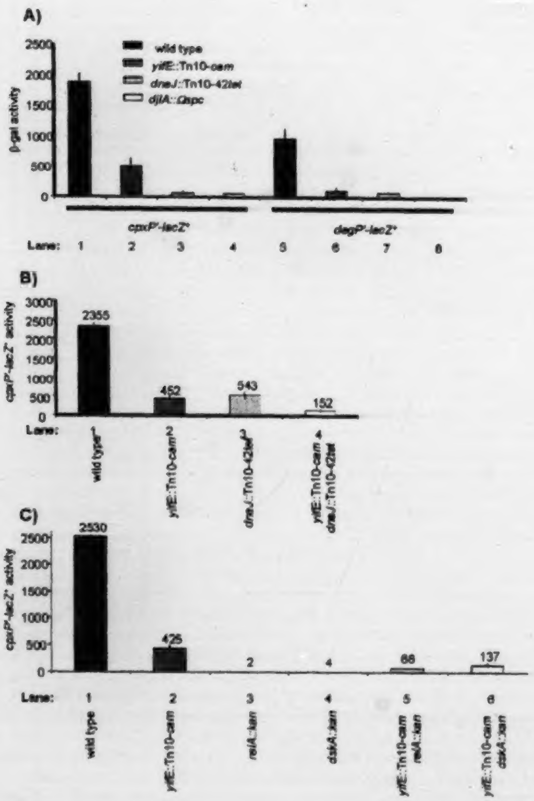

Figure 4-4. Expression of Cpx regulated genes is influenced by other cytoplasmic factors in addition to YifE. A) *cpxP'-lacZ*$^+$ or *degP'-lacZ*$^+$ fusions were used to measure gene expression after overnight growth in wild type (TR50 and TR49)(lanes 1 and 5), *yifE*::Tn10-*cam* (DB19 and DB20) (lanes 2 and 6), *dnaJ*::Tn10-42*tet* (DB141 and DB142)(lanes 3 and 7), and *djlA*::Ωspc (DB137 and DB138) (lanes 4 and 8) backgrounds. B) YifE and DnaJ are not in the same pathway. β-galactosidase levels were measured from a *cpxP'-'lacZ*$^+$ fusion in wild type (TR50)(lane 1), *yifE*::Tn10-*cam* (DB19)(lane 2), *dnaJ*::Tn10-42*tet* (DB141)(lane 3), and *yifE*::Tn10-*cam dnaJ*::Tn10-42*tet* double mutant (DB162)(lane 4) backgrounds in late stationary phase. C) Lack of ppGpp affects expression of the Cpx regulon independently YifE. β-galactosidase activity produced from a *cpxP'-lacZ*$^+$ fusion was measured after overnight growth in wild type (TR50) (lane 1), *yifE*::Tn10-*cam* (DB19) (lane 2), *relA*::kan (DB288) (lane 3), *dksA*::kan (DB289) (lane 4), *yifE*::Tn10-*cam relA*::kan (DB292)(lane 5), and *yifE*::Tn10-*cam dksA*::kan (DB293) (lane 6) mutant strains. Experiments were performed in triplicate and the means and standard deviations are presented.

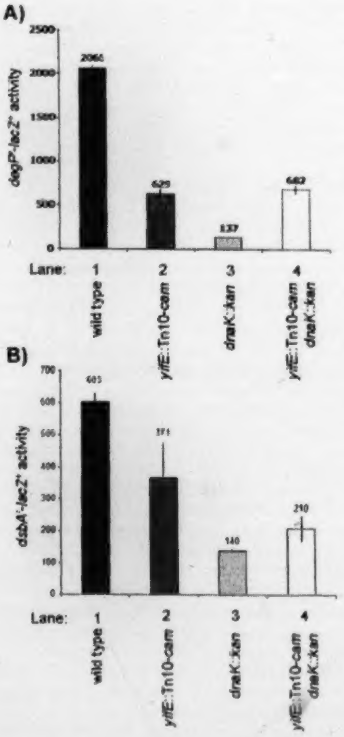

Figure 4-5. DnaK affects expression of Cpx regulon members. A) Expression of the Cpx regulon gene *degP* is affected by DnaK, which is dependent on YifE. β-galactosidase levels were measured from a *degP'-'lacZ*$^+$ transcriptional fusion in wild type (TR49) (lane 1), *yifE*::Tn10-*cam* (DB20) (lane 2), *dnaK*::kan (DB352) (lane 3), and *yifE*::Tn10-*cam dnaK*::kan (DB354) (lane 4) backgrounds in late stationary phase. B) Expression of *dsbA*, a member of the Cpx regulon, is affected by DnaK. β-galactosidase levels were measured from a *dsbA'-lacZ*$^+$ transcriptional fusion in wild type (TR96) (lane 1), *yifE*::Tn10-*cam* (DB23) (lane2), *dnaK::kan* (DB353) (lane 3), and *yifE*::Tn10-*cam dnaK::kan* (DB355) (lane 4) backgrounds in late stationary phase. Assays were performed in triplicate and the means and standard deviations are presented.

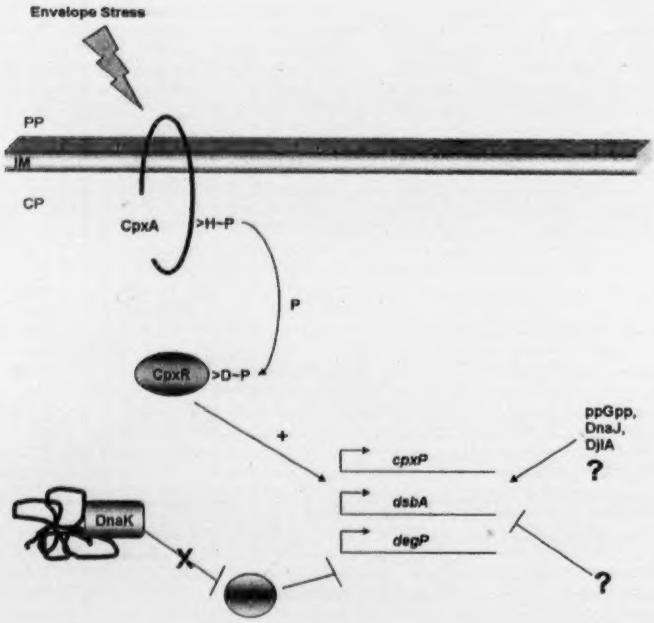

Figure 4-6. Model of how YifE inhibits expression of Cpx regulon genes. Under non-stressful conditions YifE is inhibited by DnaK either directly or indirectly. In the presence of cytoplasmic misfolded proteins, DnaK is titrated away from YifE, relieving its inhibition on YifE. YifE is now able to work as a DNA-dependent transcriptional regulator inhibiting the expression of Cpx regulated genes directly or through additional factors. YifE inhibition of Cpx regulated genes does not affect the normal signal transduction of the Cpx pathway. In addition to YifE there are additional activators, ppGpp, DjlA, and DnaJ that affect Cpx regulon members. PP, periplasm, IM, inner membrane, CP, cytoplasm, H, histidine, P, phosphate, D, aspartate.

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Chapter 5:
Conclusions and Future Directions

5.1 A highly conserved predicted N-terminal α-helix is important for CpxP inhibitory activity.

The main purpose of my research for the last 5 years has been to characterize CpxP with respect to its role in the signal transduction of the Cpx pathway. As mentioned before CpxP has no informative homologues, so we really had no information to go on with respect to domains that might be important and how CpxP might function. We initially identified a predicted N-terminal α-helix in CpxP that was highly conserved throughout other bacteria, even though the remainder of the N-terminal region is not highly conserved (Figure 2-5 and 5-2). Further characterization of this predicted α-helix showed that it was important for the ability of CpxP to mediate pathway inhibition, as well as for CpxP stability (Figure 2-2 and 2-3). Through α-helical wheel analysis (PepTool, Biotool Inc.), it was noticed that the loss-of-function mutations that affected only inhibition, such as CpxPD_{61}E, were localized to one side of the helix, while those that affected both inhibition and stability, such as CpxPQ_{55}P, were localized to the other side of the helix (Figure 5-1). Thus, it may be that the side of the helix that is involved only in inhibition is on the outside of CpxP, where it may be involved in interactions with other proteins, while the other side of the helix that is involved in stability may be oriented towards the interior of the protein, where amino acid interactions occur that are important for CpxP folding and stability. This predicted α-helix is polar except for CpxPL_{51}, CpxPL_{62} and CpxPA_{66}, suggesting that it is oriented on the outside of the protein. Thus, we propose that this α-helix is involved in interactions with the sensing domain of CpxA and/or other CpxP proteins and/or misfolded proteins.

5.2 DegP mediates changes in CpxP stability differentially in response to inducing cues.

Since some of the CpxP mutants had altered protein stability, we wanted to identify a protease that might be responsible for this. We showed that DegP was responsible for degrading CpxP (Figure 2-4 and 3-2, Table 3-2). It was observed that in the absence of DegP, CpxP was stable as measured by half life determination, and that there was a decrease in Cpx pathway activity (Figure 2-4, Table 3-2). This makes sense as an accumulation of CpxP would be expected to down-regulate the Cpx response. We also observed that DegP was needed for the proper activation of the Cpx pathway, as measured in activation experiments (Figure 3-2). In the absence of DegP, but in the presence of inducing cues, we propose that the accumulation of CpxP competes with misfolded proteins for the sensing domain of CpxA, thus preventing full activation of the Cpx pathway.

Further analysis of DegP proteolysis of CpxP revealed that there are two separate mechanisms for activating the Cpx pathway. Some inducing cues (pH, overexpression of BfpA, PapG and PapE) caused CpxP to be degraded by DegP (Figure 2-4, Table 3-2). Conversely, overexpression of NlpE did not affect the stability of CpxP (Table 3-2). It has been proposed that NlpE contains a serine endoprotease inhibitor moftif (Snyder *et al.*, 1995). Thus, overexpression of NlpE may inhibit DegP, preventing degrading CpxP. Alternatively, NlpE has been shown to be an accessory factor that the Cpx pathway needs to sense adhesion to abiotic surfaces (Otto & Silhavy, 2002). Thus, the signal generated by NlpE with respect to adhesion may be distinct from those generated by the other inducing cues, eliminating the need for CpxP to be degraded. Additionally, since the signal generated by adhesion is not well characterized, we can not rule out the idea that CpxP may play a role in adapting to this changing environment; hence that is why it is not degraded.

The phenotype of the CpxPQ$_{128}$H LOF mutant led to the hypothesis that the C-terminal region of CpxP is important for DegP-dependent proteolysis since CpxPQ$_{128}$H'-'Bla is able to inhibit the Cpx response in a *degP* null but not in a wild type background (Figure 3-6). This suggests that CpxPQ$_{128}$H may be locked in the activated form where a DegP cleavage site is always exposed and therefore CpxPQ$_{128}$H is preferentially degraded by DegP in the absence of inducing cues.

5.3 CpxP is an α-helical dimer that undergoes a conformational change at alkaline pH.

In addition to using genetics to understand the role CpxP plays in Cpx signal transduction, we also started to analyze CpxP biochemically in collaboration with Mark Glover's lab in the Department of Biochemistry. We were able to show that CpxP forms a dimer *in vivo* and *in vitro* (Figure 3-3 and 3-4). Cross-linking experiments with CpxP LOF mutants showed that the CpxPD$_{61}$E mutant was compromised in its ability to dimerize but CpxPQ$_{55}$P could still dimerize like the wild-type protein (Figure 3-7). We speculate that since CpxA likely functions as a dimer, CpxP would also need to be in a dimer form to properly interact with the CpxA sensing domain and efficiently inhibit the Cpx pathway. Thus, the CpxPD$_{61}$E mutant might be defective in inhibition of the Cpx response because of its diminished ability to dimerize. Conversely, CpxPQ_{55}P is able to dimerize in a similar fashion to wild type. We propose that the change from a hydrophillic amino acid to a hydrophobic amino acid in this mutant changes the overall folding of CpxP, thus preventing it from functioning correctly in a *degP* null but causes it to be degraded in the wild type background.

We also showed that CpxP undergoes a conformational change in response to alkaline pH (Figure 3-4 and 3-5). We propose that CpxP may change from a form where it protects the sensing domain of CpxA from false activation to a form where it is readily degraded by DegP, either alone or in conjunction with misfolded proteins. Additionally, since CpxP is needed for efficient activation of the Cpx response (Figure 3-1), we hypothesize that this conformational change may enhance or be a trigger for the conformational change that CpxA must undergo in response to inducing cues.

5.4 CpxP functions in both the inhibition and activation of the Cpx response.

One of the biggest questions that needed to be answered with respect to CpxP in Cpx signal transduction was what role it played under physiological conditions. Work done to date to characterize CpxP has relied on artificial conditions, where either CpxP or misfolded proteins were overexpressed or the Cpx pathway was constitutively activated (Raivio *et al.*, 2000, Raivio *et al.*, 1999, Buelow & Raivio, 2005, Isaac *et al.*, 2005). As part of my research, we developed a physiologically relevant assay to look at the affects CpxP has on the initial activation and shut-off of the Cpx pathway. This assay allowed us to show that CpxP was important for both the activation and repression of the pathway (Figure 3-1). We hypothesized that CpxP was important for shutting down the Cpx pathway, since CpxP was initially characterized as an inhibitory protein (Raivio *et al.*, 2000, Raivio *et al.*, 1999). However, the finding that CpxP was important in the activation of the Cpx pathway was surprising. The role CpxP plays in the activation of the Cpx pathway may be by protecting the sensing domain of CpxA from background noise caused by normally occurring misfolded proteins, thus preventing the false activation of the Cpx pathway. Thus, when CpxP is absent the CpxA kinase is "set" at a higher level, where it requires a larger inducing signal in order to respond. Alternatively, the requirement of CpxP for activation may reflect a conformational change that CpxP undergoes that is a trigger that causes CpxA to switch from a phosphatase to an autokinase. Thus, we speculate that CpxP is working as a fine tuning accessory factor, rather then just an inhibitory protein of the Cpx pathway.

5.5 A model for the role of CpxP in Cpx signal transduction.

In the absence of envelope stress, we propose that CpxP could protect the sensing domain of CpxA under normal conditions from background noise, allowing it to be sensitive to the presence of elevated levels of misfolded or mislocalized proteins (Figure 3-1 and 3-8). CpxP may initially sense envelope stress via misfolded or mislocalized proteins, causing it to adapt a more compact (activated) formation. Alternatively, the stresses that cause envelope proteins to become misfolded may also cause CpxP to change conformation. This conformational change could cause a corresponding conformational change in CpxA, priming it to respond to the stresses present. Thus, explaining the role of CpxP in activation of the response (Figure 3-1). The activated conformation of CpxP may allow it to bind to misfolded proteins and target them to DegP. Alternatively, the activated conformation could target CpxP independently to DegP. In either case, we hypothesize that this CpxP conformational change exposes a region within the C-terminal end of the protein that is involved in targeting CpxP for DegP dependent proteolysis (Figure 3-8). The presence of misfolded proteins/envelope stress would maintain CpxP in this compact activated conformation rendering it unstable until the stress has been cleared.

After envelope stress has been relieved, CpxP might change back to its normal flatter formation, halting its degradation and signaling to CpxA, possibly through a direct interaction that may be facilitated through the N-terminal region of CpxP, to shut off the pathway (Figure 4-2). We hypothesize that the role CpxP plays in the activation and inhibition of the Cpx pathway requires it to be a dimer and in the absence of dimerization CpxP is unable to carry out its proper function. Although CpxP is a third component of the Cpx pathway, it is dispensable, unlike CpxA and CpxR. The Cpx pathway can still be fully activated in response to inducing cues and shut off when CpxP is not present. However the overall response time is extended when CpxP is absent (Figure 3-1), thus CpxP allows the Cpx pathway to be more efficiently activated and shut off. We speculate that the efficiency of activation and shut off when CpxP is present could make the bacteria fitter to withstand changing environments, ultimately yielding an evolutionary advantage.

5.6 The expression of the Cpx regulon is influenced not only by envelope stress but also cytoplasmic stresses and factors.

Stress responses have been compartmentalized into the cytoplasmic and extracytoplasmic (envelope) stress responses. While the major role of the extracytoplasmic stress responses is to monitor the integrity of the envelope, it has been shown with the σ^E pathway that it can also respond to cytoplasmic stress signals, such as ppGpp (Costanzo & Ades, 2006). In addition Button *et al.* (2007) showed that a putative DNA-binding protein, YdcQ, affects expression of Cpx regulated genes independently of CpxRA. We also showed that the Cpx pathway is influenced by cytoplasmic factors (Chapter 4). In addition to ppGpp controlling σ^E activity upon entry into stationary phase, we found that when ppGpp production was eliminated there was a decrease in Cpx gene expression (Figure 4-4). Additionally, we showed that DnaJ and DjlA, cytoplasmic chaperones, affected the Cpx pathway (Figure 4-4). While the mechanism for how ppGpp, DnaJ and DjlA affect Cpx pathway activity is not known, our results, together with Costanzo and Ades (2006) and Button *et al.* (2007), suggest that a common theme in envelope stress response regulation is some level of communication between the cytoplasm and envelope that helps coordinate the response to changing environments.

We also identified a transcriptional regulator, YifE, and a heat shock chaperone, DnaK, that affected activity of the Cpx regulon. We showed that a gain-of-function *yifE*::Tn10-*cam* mutation, and overexpression of YifE from an exogenous promoter, negatively affected the regulation of the Cpx regulon, while a *yifE::kan* mutant did not affect expression (Figure 4-1). This suggests that YifE is a stress response regulator, since at the basal level of gene expression YifE has no effect. We hypothesize that the gain-of-function phenotype seen with *yifE*::Tn10-*cam* is due to a loss in regulation of the protein by the disruption of the C-terminal region of YifE by the Tn10 element. The negative affect YifE had on the Cpx pathway was most prevalent in stationary phase (Figure 4-2). In addition we showed that DnaK and YifE may function in the same pathway (Figure 4-5). One model posits that normally DnaK inhibits YifE, either directly or through additional factors that have yet to be discovered. However, in the presence of cytoplasmic misfolded proteins, DnaK is titrated away from YifE in conjunction with the misfolded proteins, relieving the inhibition on YifE (Figure 4-6). YifE is now able to negatively influence expression of the Cpx and other envelope stress regulons. The affect YifE had on Cpx regulated gene expression was not limited to the Cpx pathway. We showed that YifE is a general regulator that works both as an activator and a repressor (Table 4-3). We hypothesized that the role of YifE is to help conserve energy when cytoplasmic misfolded proteins are present. This response would allow the bacteria to devote more resources to eliminating the stress at hand.

Although YifE influences Cpx gene expression, it does not affect the signal transduction of the pathway, because in the presence of inducing cues the Cpx pathway is still induced to a similar level in both wild type and *yifE::*Tn10-*cam* strains (Figure 4-2). We hypothesize that when additional stresses are encountered they override the negative regulation imposed by YifE. Thus, bacterial survival is not compromised. Notably, YifE is a higly conserved, completely uncharacterized protein. Thus, our findings are also significant in that they ascribe a function to this new regulator.

5.7 Questions that remain.

5.7.1 Does CpxP inhibit DegP activity?

It has been hypothesized that the glutamine rich region of the periplasmic domain of RseA interacts with the periplasmic PDZ domain of RseP, inhibiting RseA degradation by RseP until an appropriate stress has been sensed (Kanehara *et al.*, 2003). RseA must undergo sequential degradation, first by DegS and then by RseP in response to misfolded proteins (Kanehara *et al.*, 2003, Ades *et al.*, 1999, Alba *et al.*, 2002). This series of events initially leads to the release of the glutamine rich periplasmic domain of RseA, thus relieving the inhibition of RseP, allowing it to cleave RseA.

Interestingly it has been observed that CpxP is rich in glutamine residues (19/130). Comparison of a number of CpxP homologues showed that many of the glutamine residues in the C-terminal region are highly conserved (9/11)(Figure 5-2). In addition one LOF CpxP mutant, CpxPQ$_{128}$H, specifically affected the stability of CpxP (Figure 3-6). Thus, given the role of the glutamine rich region of RseA in inhibiting RseP, it may be that the C-terminal region of CpxP inhibits one of the two PDZ domains of DegP under non-inducing conditions. Interestingly, we showed a conformational change in CpxP in response to alkaline pH (Figure 3-4 and 3-5) and we noted that CpxP was stable at pH 5.8 but not 8.0 (Table 3-3, Figure 2-4). Thus, it may be that the flatter conformation CpxP takes on at pH 5.8 exposes more of the C-terminal glutamine residues, thus inhibiting DegP. However, at pH 8.0, when CpxP takes on a more compact conformation, the C-terminal glutamine residues may be hidden from the surface of CpxP, thus eliminating the inhibition CpxP may exert on DegP.

Testing this model experimentally would be quite difficult as it would involve the mutation, individually and possibly in combination, of all of the conserved glutamine residues in CpxP, followed by an analysis of protein stability. However, determining the crystal structure of CpxP at both pH 5.8 and 8.0 might allow insight into how these glutamine residues are situated within CpxP. If it is observed that more glutamine residues are present on the surface of CpxP at pH 5.8 then 8.0, we could hypothesize that they do aid in preventing degradation by DegP. In addition, if the crystal structures show a difference in the positions of glutamines on the surface of CpxP at pH 5.8 and 8.0 it may be interesting to try and create mutants that would lock CpxP in either the repressed (flatter) or activated (compact) conformation like CpxPQ_{128}H. We hypothesize that CpxPQ_{128}H may be locked in the activated conformation where it is always degraded. Once these mutants are isolated one could look at the degradation profile of such mutants in response to inducing cues to identify residues important for DegP inhibition.

5.7.2 Where is the DegP cleavage site in CpxP?

We have shown that CpxP is degraded by DegP in response to numerous Cpx inducing cues and that under normal conditions DegP is responsible for the natural turn over of CpxP (Table 3-3). Thus, there must be DegP cleavage sites within CpxP. It has been shown that DegP cleaves at either a V/X or I/X site (Kolmar *et al.*, 1996). This suggests that a hydrophobic side chain in the P1 position is important for degradation by DegP. Within CpxP there are nine V/X sites and two I/X sites (Figure 5-2). However from degradation studies of PapA by DegP (Jones *et al.*, 2002), we know that there must be additional factors besides the hydrophobic side chain at the P1 position since not all the V/X or I/X sites within PapA are cleaved by DegP. Thus, the 11 potential sites within CpxP are probably not all targets of DegP. Within CpxP there are four potential V/X sites and one I/X site that are highly conserved (Figure 5-2). Thus, it would be interesting to investigate further if any of these sites were potential DegP cleavage sites. One could subject any one of the numerous overexpression vectors that contain CpxP to site directed mutagenesis (SDM) to alter the highly conserved valine and isoleucine residues in CpxP and then look at the degradation profile by DegP in response to inducing cues. In the Jones *et al.* (2002) study they noticed that if they changed the V/V cleavage site to S/S, DegP dependent degradation was abolished. Thus, initially changing the highly conserved valines or isoleucines within CpxP to serine would give one some idea as to a potential DegP cleavage site. Peptide scanning revealed that there were three regions within PapA that were susceptible to DegP cleavage (Jones *et al.*, 2002). Thus, simple SDM may not alleviate proteolysis of CpxP by DegP. As the mature CpxP is only 130 amino acids, carrying out peptide scanning of 7 or 12 mer peptides (Jones *et al.*, 2002) for potential DegP cleavage sites might be easier to do. It would also give one an idea as to how many regions within CpxP are cleaved by DegP and where they fall. After determining this it would be easier to do SDM on the native CpxP to specifically localize the cleavage sites and verify the peptide scanning.

5.7.3 Is there a difference between the CpxP dimerization and inhibition domains?

We identified three classes of CpxP LOF mutants that affected either stability, inhibition or both stability and inhibition (Chapters 2 and 3). Characterization of these mutants showed that CpxPD_{61}E lost its ability to inhibit the Cpx pathway in both a wild type and *degP* null background and had compromised ability to dimerize (Figure 3-6 and 3-7). This suggested that CpxPD_{61}E may not be able to inhibit the pathway because of an inability to dimerize. Interestingly, a LOF mutant that retains an intact N-terminal domain, CpxPQ_{128}H, maintains the ability to inhibit the Cpx response in a *degP* null background supporting the conclusion that the N-terminal region is important for inhibition. Not all mutations that fall in this N-terminal domain influence dimerization, as the CpxPQ_{55}P mutant exhibited wild type levels of dimerization (Figure 3-7). Thus, it may be that the N-terminal domain is important not only in dimerization, it may have other functions in inhibiting the pathway. For example it could be involved in interacting with CpxA or misfolded proteins.

The Cpx pathway has been reconstituted *in vitro*. These studies showed that when CpxP was present there was a down regulation of the autokinase activity of CpxA (Fleischer *et al.*, 2007). In addition Konkel *et al.* (2005) have determined which region of the adhesion lipoprotein, CadF, from *Campylobacter jejuni* is important in binding fibronectin, using overlapping 30 or 16 mer peptides from CadF. They showed that a four amino acid patch within CadF was responsible for binding fibronectin (Konkel *et al.*, 2005). Thus, it would interesting to generate such a peptide library for CpxP and use it in proteoliposomes that contain only CpxA to determine if there is any specific region within CpxP that affects the autokinase activity of CpxA. Such a study might allow for the identification of individual functional domains within CpxP responsible for dimerization and CpxA interaction.

5.7.4 Do CpxP and CpxA interact?

The simplest model for how CpxP inhibits the Cpx response proposes a direct interaction between CpxP and CpxA. Despite the long-standing nature of this prediction, this part of the model remains to be characterized.

I used a CpxP'-'Bla fusion protein and a GST (glutathione S transferase) tag fused to the sensing transmembrane domains of CpxA to see if I could detect a difference in Cpx pathway activity when GST-CpxA was present in the cell. We predicted that if CpxP interacted with the sensing domain of CpxA, adding additional CpxA sensing domains without the kinase domain would cause a shift in the number of interactions between CpxP and the native CpxA, which would be reflected in activity of the Cpx pathway (Figure 5-4). One would predict that if CpxP is titrated away from the native CpxA by the GST-CpxA fusion protein, there would be an increase in Cpx pathway activity. This change in pathway activity should be negated when CpxA is removed. However if CpxP is not titrated away from the native CpxA in the presence of the GST-CpxA fusion protein, there would be no change in Cpx pathway activity. When the GST-CpxA fusion was co-expressed with the CpxP'-'Bla protein there was an increase in pathway activity (Figure 5-5, compare lanes 1 and 2), suggesting that CpxP'-'Bla was being titrated away from the native CpxA to interact with the GST-CpxA fusion. However, when the same experiment was performed in a *cpxA* null strain, there was no change in Cpx pathway activity (Figure 5-5, compare lane 5 and 6). This genetic data tentatively suggests that there is an interaction between the sensing domains of CpxA and CpxP. However, this conclusion still needs to be verified in a different way. I have tried to verify the results from the titration assay with cross-linking experiments, pull down assays, protease K protection assays, and cell fractionation experiments. However, none of these experiments were conclusive (data not shown).

Since the N-terminal region of CpxP, where we isolated LOF mutations, is highly conserved, we hypothesized that there may be conserved regions within CpxA that are responsible for a possible interaction with CpxP. However, sequence comparisons show that the whole sensing domain of CpxA is highly conserved (data not shown). Thus, identifying potential regions within CpxA that may interact with CpxP by sequence gazing is impossible. However we observed that the predicted secondary structure of the CpxA sensing domain is similar to that of EnvZ, CitA and DcuS (Figure 5-6), with a central β-sheet configuration. This topology is indicative of a PAS domain (Khorchid & Ikura, 2006).

PAS domains have been shown to be important in protein-protein interactions (Taylor & Zhulin, 1999). Until recently they were thought to be involved in intracellular functions, but with the identification of PAS domains in the sensing domains of CitA, DcuS and PhoQ, it is now clear that they are also involved in extracellular signaling (Cano *et al.*, 2002, Reinelt *et al.*, 2003, Pappalardo *et al.*, 2003). Thus, we hypothesize that a potential PAS domain within CpxA may be involved in protein-protein interactions. These interactions may be with CpxP and/or misfolded proteins that the pathway senses.

Interestingly, the sensing domains of CitA, PhoQ and DcuS have been shown to be water-soluble (Cho *et al.*, 2006, Reinelt *et al.*, 2003, Pappalardo *et al.*, 2003). Thus, it may be possible to express the sensing domain of CpxA, instead of the entire CpxA protein, and look for a possible interaction with CpxP. We recently used the *Bordetella pertussis* adenylate cyclase two-hybrid system (Karimova *et al.*, 1998) to show that CpxP forms dimers *in vivo* (Figure 5-7). We cloned *cpxP* minus its signal sequence into vectors containing either the T25 or T18 domains of the *B. pertussis* CyaA protein. These vectors were expressed in various combinations in an *E.coli cya*$^-$ strain and cAMP production was assayed by measuring β-galactosidase activity. We observed that an N-terminal CyaAT25'-'CpxP fusion protein was able to interact with both an N-terminal CyaAT18'-'CpxP and a C-terminal CpxP'-'CyaAT18 fusion proteins (Figure 5-7, lanes 5 and 8). However in the presence of a CpxP fusion protein and the vector control there was little β-galactosidase activity (Figure 5-7, lanes 3, 4, 6, and 7), suggesting that the increase activity seen when two CpxP fusion proteins are present is specific to a CpxP-CpxP interaction. Thus, since it seems that CpxP retains the ability to form dimers in the cytoplasm (Figure 5-7),we hypothesize that the periplasmic sensing domain of CpxA may also be functional and interact with CpxP within the cytoplasm. Accordingly, one could clone the sensing domain of CpxA into the *B.pertussis* T25 and T18 vectors and express the resultant fusion proteins in combination with the relevant CpxP constructs to determine if an interaction between CpxP and CpxA exists by analyzing production of β-galactosidase. Also, since this assay would be *in vivo*, manipulation of growing conditions would be rather easy to determine an optimal condition when CpxP and CpxA interact. In addition, if one can clone just the sensing domain of CpxA to a tag, such as GST, there is a possibility that one could retry the pull down experiments to try and identify a potential interaction between CpxP and CpxA. Showing that CpxA and CpxP interact would confirm the long-standing speculation that they do interact and also open the door to further characterization of the Cpx signaling cascade.

Table 5-1. Strains used in chapter 5.

Strains	Description	Reference
MC4100	F^- *araD139* Δ(*argF-lac*)U169 *rspL150* (Str^R) *relA1 flbB5301 decC1 ptsF25 rbsR*	(Casadaban, 1976)
BTH101	F^- *cya*$^-$*99 araD139 galE15 galK16 rpsL1* (Str^R) *hsdR2 mcrA1 mcrB1*	D. Landant
TR50	MC4100 λRS88 (*cpxP'-lacZ*$^+$)	(Raivio *et al*., 1999)
DB152	TR50(pGEX)(pBBR1MCS)	This study
DB153	TR50 (pGA)(pBBR1MCS)	This study
DB154	TR50 (pGEX)(pPB)	This study
DB155	TR50 (pGA)(pPB)	This study
DB276	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *cpxA::kan*	This study
DB284	DB276 (pGEX)(pBBR1MCS)	This study
DB285	DB276 (pGEX)(pPB)	This study
DB286	DB276 (pGA)(pBBR1MCS)	This study
DB287	DB276 (pGA)(pPB)	This study
DB378	BTH101 (pKT25-zip)(pUT18-zip)	This study
DB379	BTH101 (pKT25-CpxP)(pUT18C)	This study
DB380	BTH101 (pKT25)(pUT18C-CpxP)	This study
DB381	BTH101 (pKT25-CpxP)(pUT18C-CpxP)	This study
DB382	BTH101 (pKT25)(pUT18-CpxP)	This study
DB383	BTH101 (pKT25-CpxP)(pUT18)	This study
DB384	BTH101 (pKT25-CpxP)(pUT18-CpxP)	This study

Table 5-2. Plasmids used in chapter 5.

Plasmids	Description	Reference
pBBR1MCS	Expression vector with a *lac* promoter (Cam^R)	(Kovach *et al.*, 1994)
pGEX-2T	Expression vector for N-terminal GST fusion proteins (Amp^R)	Amersham
pGA	GST-CpxA sensing domain translational fusion vector	This study
pPB	CpxP'-'Bla overexpression vector (Amp^R,Cam^R)	(Buelow & Raivio, 2005)
pKT25	Bacterial two hybrid plasmid containing the *cyaA*T25 domain, used for N-terminal fusion proteins (Kan^R)	D. Ladant; (Karimova *et al.*, 1998)
pKT25-CpxP	CyaAT25'-'CpxP cytoplasmic translational fusion vector	This study
pKT25-zip	Bacterial two hybrid control plasmid containing a leucine zipper from the yeast transcriptional regulator, GCN4 (Kan^R)	D. Ladant; (Karimova *et al.*, 1998)
pUT18	Bacterial two hybrid plasmid containing the *cyaA*T18 domain, used for N-terminal fusion proteins (Amp^R)	D. Ladant; (Karimova *et al.*, 1998)
pUT18-CpxP	CyaAT18'-'CpxP cytoplasmic translation fusion vector	This study
pUT18-zip	Bacterial two hybrid control plasmid containing a leucine zipper from a yeast transcriptional regulator, GCN4 (Amp^R)	D. Ladant; (Karimova *et al.*, 1998)
pUT18C	Bacterial two hybrid plasmid containing the *cyaAT18* domain, used for C-terminal fusion proteins (Amp^R)	D. Ladant
pUT18C-CpxP	CpxP'-'CyaAT18 cytoplasmic translational fusion vector	This study

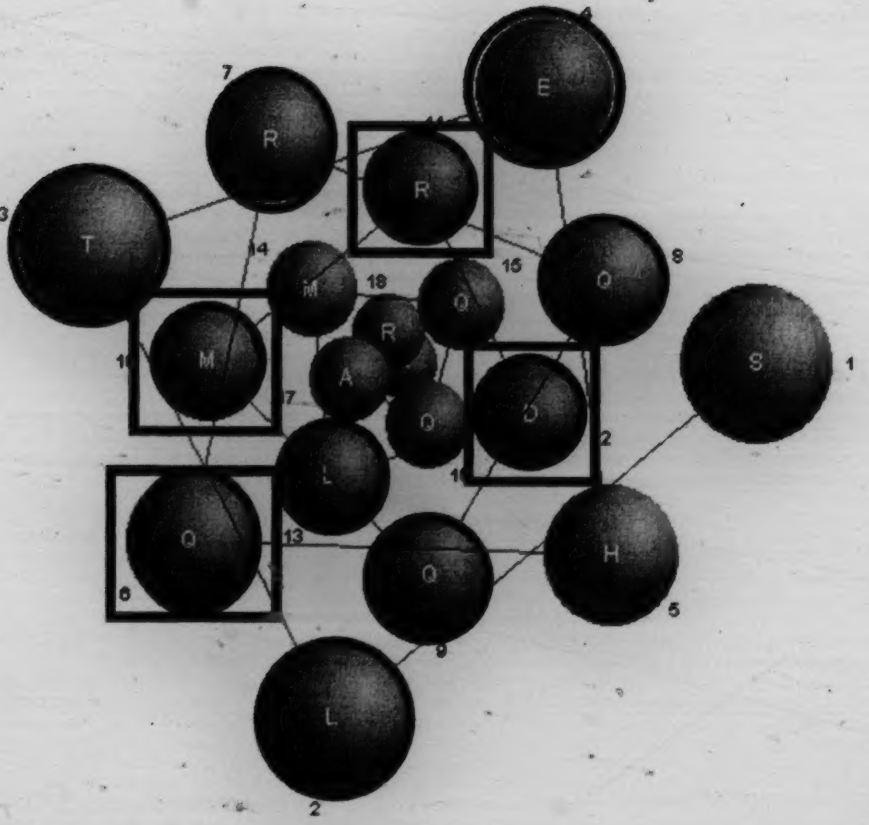


Figure 5-1. Helical wheel analysis of the predicted N-terminal α-helical domain of CpxP that is important in inhibition and stability. Blue circles represent amino acids that are predicted to be found within this α-helix. Those that contain a black circle around them are amino acids that are completely conserved in all CpxP homologues. Those with black squares around them are amino acids that, when mutated cause CpxP to loose both the ability to inhibit the Cpx response and stability. The amino acids with red squares around them are the amino acids that caused CpxP to loose inhibition but not stability. Helical wheel analysis was performed using PepTool (Biotools Inc.).

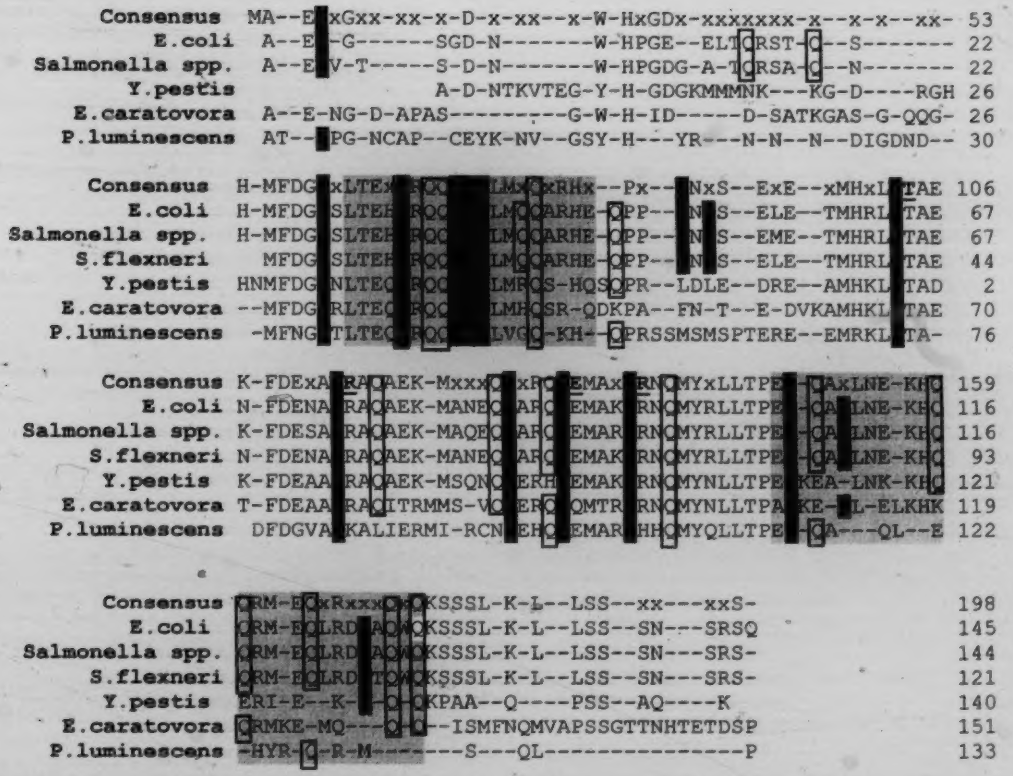


Figure 5-2. Sequence alignment of CpxP orthologues. The amino acid sequence of the mature *E.coli* CpxP was aligned with those of *Salmonella* spp., *Shigella flexneri*, *Yersinia* spp., *Erwinia caratovora,* and *Photorhabdus luminescens* using PepTool (Biotools Inc.). The consensus sequence is indicated on the top line. Glutamine (Q) residues are boxed in blue, while the conserved predicted α-helix where the six loss of function mutations localized are highlighted in yellow, with the amino acids that were mutated indicated in red. In addition, potential valine (V) and isoleucine (I) residues that may be involved in DegP proteolysis are highlighted in purple. Highly conserved V and I sites throughout CpxP orthologues are bolded and underlined.

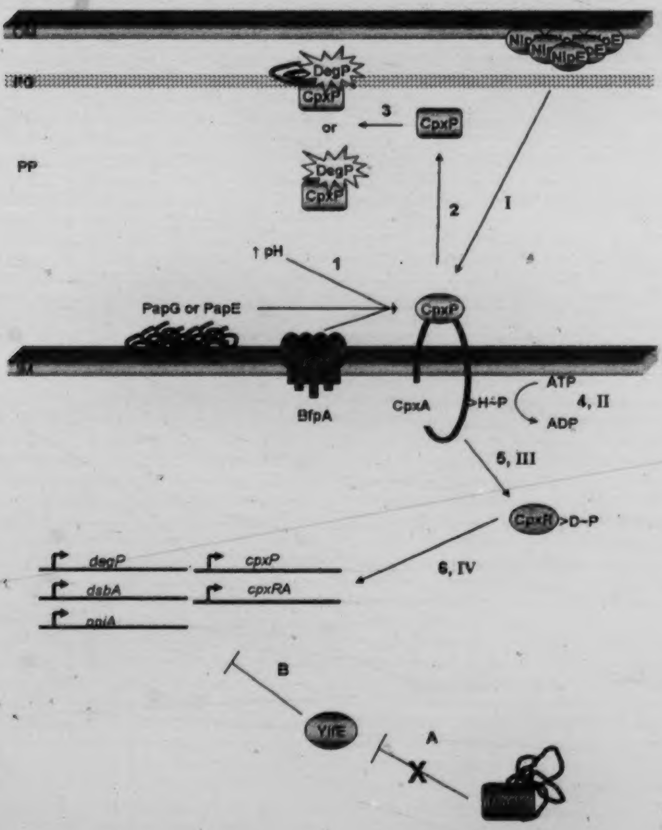


Figure 5-3. Complete model of the Cpx response. Under non-inducing conditions CpxP is hypothesized to interact with the sensing domain of CpxA via the N-terminus, downregulating the Cpx response. Under inducing conditions, such as alkaline pH or overexpression of PapG, PapE or BfpA (1), CpxP is titrated away either alone or in conjunction with misfolded proteins (2). CpxP then undergoes a confirmation change which is proposed to expose a C-terminal DegP cleavage site resulting in the DegP dependent degradation event of CpxP, which may occur alone or in conjunction with misfolded proteins (3). The sensing domain of CpxA is now free to sense the stresses present and autophosphorylate itself using ATP on a conserved histidine residue (4). This phosphate is then transferred to CpxR (5), which intern upregulates numerous protein folding and degrading factors (6). Alternatively, the Cpx pathway can be activated by NlpE, which bypasses DegP dependent degradation of CpxP (I), but still propagates a phosphotransfer event between CpxA and CpxR, allowing CpxR to act as a transcriptional regulator (II, III and IV). Additional Cpx activity is influenced by cytoplasmic factors. During stationary phase, DnaK senses misfolded proteins, which relieves the inhibition it exerts on the transcriptional regulator, YifE (A). YifE now is able to work as a global regulator affecting the regulation of numerous genes, more specifically it negatively regulates the Cpx regulon (B), but does not affect the signal transduction of the Cpx pathway. OM, outer membrane, PP, periplasm, IM, inner membrane, PG, peptidoglycan lalyer, H, histidine, D, aspartic acid, P, phosphate.

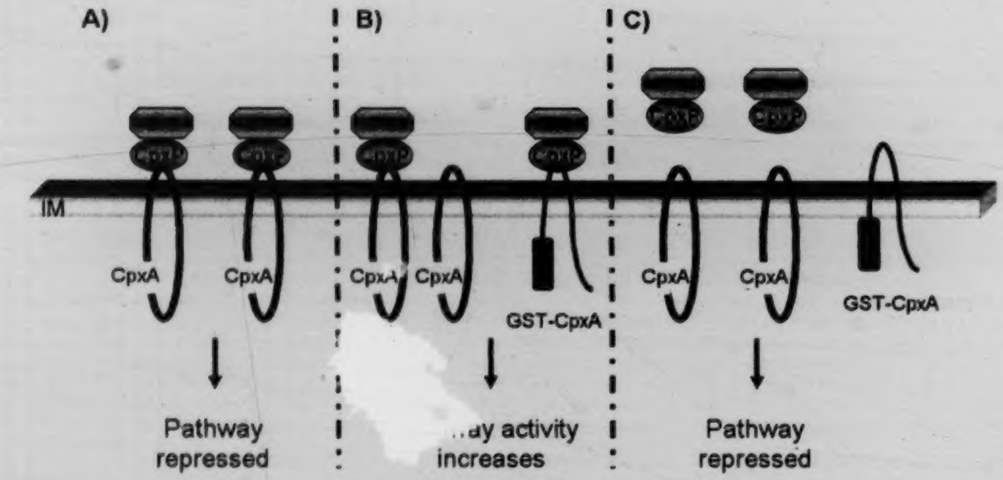

A)
B)
C)
IM
CpxA
CpxA
CpxA
CpxA
GST-CpxA
CpxA
CpxA
GST-CpxA
Pathway repressed
..ay activity increases
Pathway repressed

Figure 5-4. Strategy and predictions of the titration assay between CpxP and GST-CpxA. CpxP'-'Bla is overexpressed from pPB. While GST-CpxA is overexpressed from pGA. A) In the presence of CpxP overexpression, it is hypothesized that CpxP interacts with the sensing domain of CpxA, causing the Cpx pathway to be repressed. B) If CpxP interacts with the sensing domain of CpxA, we hypothesize that the addition of an additional CpxA sensing domain would cause CpxP to be titrated away from the native CpxA by binding to the GST tagged CpxA sensing domain. This would leave some of the native CpxA free, resulting in an increase in pathway activity. C) If CpxP does not interact with the sensing domain of CpxA, the addition of an additional CpxA sensing domain would have no affect on pathway activity. GST, glutathione S transferase, IM, inner membrane

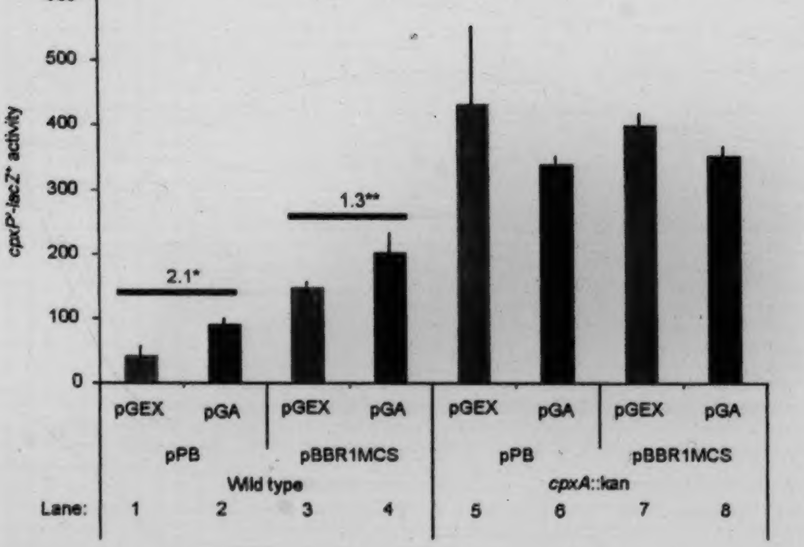


Figure 5-5. Activity of the Cpx pathway in the presence of an additional CpxA sensing domain and overexpression of CpxP'-'Bla in a wild type or *cpxA*::kan background. Pathway activity was measured using a chromosomal *cpxP'-lacZ*$^+$ reporter inserted in the λRS88 site. The additional sensing domains of CpxA were fused to a GST tag and carried on pGA. The vector control for pGA is pGEX. Overexpression of CpxP'-'Bla occurred via pPB. The vector control for pPB is pBBR1MCS. The fold difference between pathway activity in strains transformed with pGEX compared to pGA and also carrying pPB (lanes 1 and 2) is 2.1 with a P-value of 0.0056. The fold difference in strains transformed with pGEX compared to pGA and also carrying pBBR1MCS (lanes 3 and 4) is 1.3 with a P-value of 0.0309. The same vectors in a *cpxA::kan* strain exhibited equivalent levels of *cpxP'-lacZ*$^+$ expression under all conditions. Strains used are DB154 (lane 1), DB155 (lane 2), DB152 (lane 3), DB153 (lane 4), DB285 (lane 5), DB287 (lane 6), DB284 (lane 7), and DB286 (lane 8).

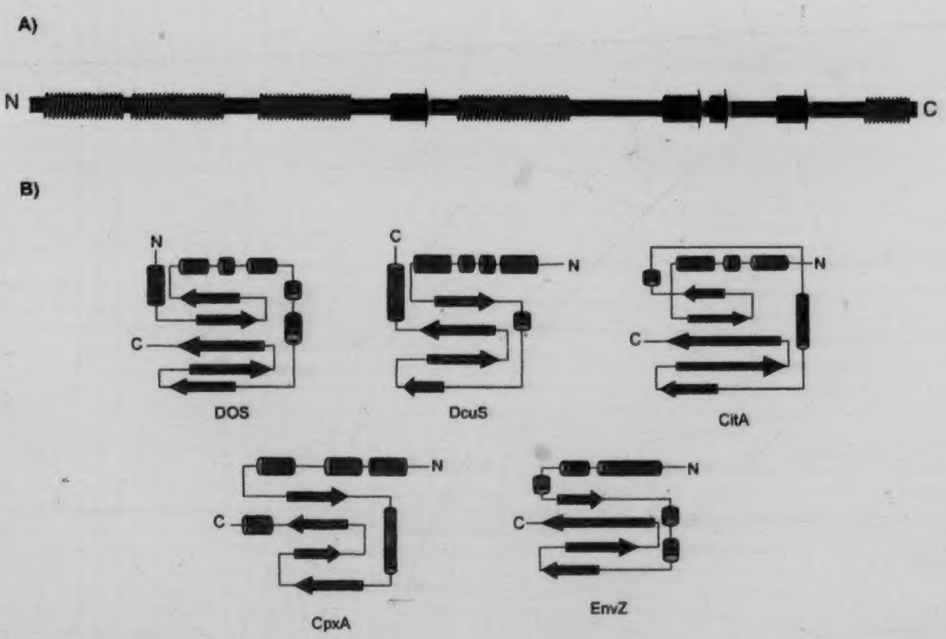
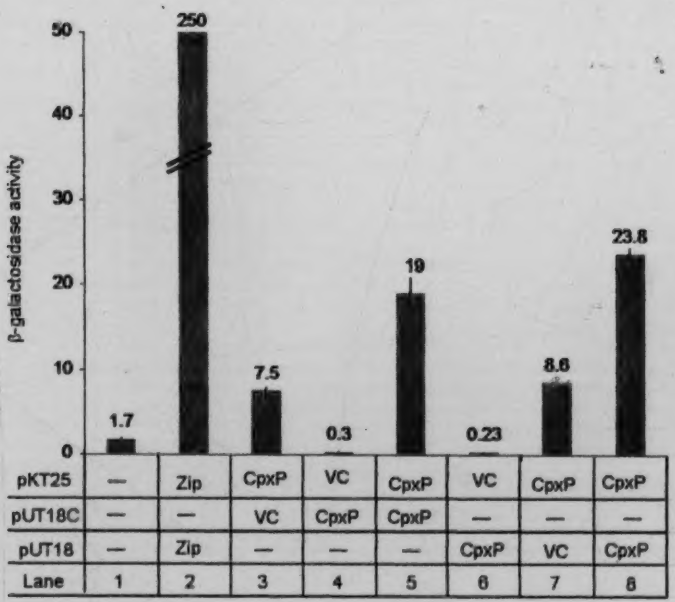


Figure 5-6. Predicted secondary structure and topology of the sensing domain of CpxA. A) Predicted secondary structure of the sensing domain of CpxA as determined using PepTool (Biotools Inc.). Arrows indicate predicted β-sheets, while coils indicate predicted α-helices. B) The predicted topologies of DOS, a phosphodiesterase , DcuS, CitA, and EnvZ were adapted from Khorchid and Ikura (2006). Based on the topologies of these sensing domains a predicted topology of the CpxA sensing domain was determined.



pKT25	—	Zip	CpxP	VC	CpxP	VC	CpxP	CpxP
pUT18C	—	—	VC	CpxP	CpxP	—	—	—
pUT18	—	Zip	—	—	—	CpxP	VC	CpxP
Lane	1	2	3	4	5	6	7	8

Figure 5-7. Bacterial two-hybrid analysis of CpxP-CpxP dimerization. Full length CpxP (lacking the signal peptide) was fused to either the either N-terminus or C-terminus of the adenylate cyclase domains, T25 or T18 of *Bordetella pertussis*. CpxP was fused in frame to either the N-terminus of the T25 or T18 domains or the C-terminus of the T18 domain and co-transformed into reporter strain, BTH101, with various constructs and vector controls. β-galatosidase activity was measured after 16 hours. Strains used were BTH101 (lane 1), DB378 (lane 2), DB379 (lane 3), DB380 (lane 4), DB381 (lane 5), DB382 (lane 6), DB383 (lane 7), and DB384 (lane 8). VC, vector control, Zip, a leucine zipper motif from the yeast transcriptional regulator, GCN4.

5.8 References.

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Appendix A

Characterization of a truncated form of CpxP*

*Done in collaboration with David C. Arthur from Mark Glover's lab in biochemistry

A.1 Introduction.

In collaboration with Mark Glover's lab in Biochemistry, we tried to crystallize purified CpxP. During the crystallization process it was determined that any CpxP crystals formed were quite fragile suggesting that the mature form of CpxP is unstable. To determine it there was a more stable form of CpxP, 5µg of CpxP was exposed to varying concentrations of trypsin. After 15 minutes digestion with 0.05µg of trypsin, CpxP was cleaved into two stable forms, a 13 kDa and 15.9 kDa product. The 15.9 kDa fragment was stable for two hours after digestion with trypsin, while the 13 kDa fragment was stable past the final time point taken at 8 hours. After MALDI-TOF mass spectrometry (Univeristy of Alberta Chemistry Facility) it was determined that the 13 kDa product was made up of CpxP from Ser_{40} to Lys_{151} (Figure A-1). While the 15.5 kDa product was made up of His_{20} to Lys_{151} (Figure A-1). We were interested in further characterizing these stable CpxP products for their ability to inhibit Cpx pathway activity.

A.2 Materials and Methods.

A.2.1 Strains and plasmids.

Strains containing pMCP, $pMCP_{20-151}$ or $pMCP_{20-151}$ were maintained with 100 µg/mL of ampicillin (Sigma). Strains carrying the *degP*::Tn10 allele were maintained with tetracycline at 30°C.

A.2.2 Construction of $pMCP_{40-151}$ and $pMCP_{20-151}$.

MBP fusions to the truncated forms of CpxP were constructed using pMal-p2 (New England Biolab). $CpxP_{20-151}$ was amplified from the chromosome of MC4100 using restriction tagged primers CpxP5'EcoR1 (5'-GGA ATT CCC ACG CTG CTG AAG TCG GTT CAG GC-3') and CpxP3'Frag (5'-CCC AAG CTT TTA TTT TTG CCA TTG CGT CAC GTC-3'), which contains a stop codon (as indicated by the underlined base pairs) . The PCR product was digested with *Eco*RI and *Hind*III and cloned into the *Eco*RI and *Hind*III sites of pMal-p2 to create an in-frame fusion between MBP and the 20th amino acid of CpxP. Using CpxP5'Frag (5'-ATT TCA GAA TTC AGT ACG CAG AGC CAT ATG TTC-3') and CpxP3'-Frag, $CpxP_{40-151}$ was amplified off of the chromosome of MC4100. The resulting PCR product was digested with *Eco*RI and *Hind*III and cloned into the *Eco*RI and

*Hind*III sites of pMal-p2 to create an in-frame fusion between MBP and the 40th amino acid of CpxP.

A.3 Results.

A.3.1 Cpx pathway activity in the presence of pMCP$_{40\text{-}151}$ and pMCP$_{20\text{-}151}$.

pMCP$_{40\text{-}151}$ and pMCP$_{20\text{-}151}$ was transformed into TR50, which carries a single copy of *cpxP'-lacZ*$^{+}$ at the λRS88 and TR757, which also carried the same *cpxP'-lacZ*$^{+}$ transcriptional fusion on the chromosome as well as a *degP*::Tn10 allele resulting in DB317, DB319 and DB318, DB320, respectively (Table A-1). Cpx pathway activity of DB317, DB318, DB319 and DB320 was assayed on Lactose MacConkey media after overnight growth and compared to the positive controls TR783, which contains the *cpxP'-lacZ*$^{+}$ transcriptional fusion and the full length version of MBP-CpxP and SS1, which also contains the transcriptional fusion, the full length version of MBP-CpxP as well as the *degP*::Tn10 allele. Strains containing the full length version of MBP-CpxP (TR783 and SS1) exhibited decreased pathway activity, as previously reported (Table A-2, rows 1 and 4) (Raivio *et al.*, 1999). Pathway activity when MBP-CpxP$_{40\text{-}151}$ was expressed was comparable to that when MBP-CpxP was overexpressed, showing a decrease in pathway activity (Table A-2, row 2 and 5). Strains carrying MBP-CpxP$_{20\text{-}151}$ showed no decrease in pathway activity (Table A-2, row 3 and 6). Thus MBP-CpxP$_{40\text{-}151}$ is able to inhibit the Cpx pathway.

A.3.2 MBP-CpxP$_{40\text{-}151}$ has similar expression levels compared to MBP-CpxP, while MBP-CpxP$_{20\text{-}151}$ is unstable.

Since MBP-CpxP$_{20\text{-}151}$ did not affect pathway activity when expressed in TR50 and TR757, we wanted to determine if the protein could be detected on a western blot. MBP-CpxP and MBP-CpxP$_{40\text{-}151}$ were both detected in the presence and absence of *degP* (Figure A-2 lanes 1, 2, 3 and 4). In addition, MBP-CpxP$_{40\text{-}151}$ did show a decrease in protein size, as expected, when compared to MBP-CpxP. Since MBP-CpxP$_{20\text{-}151}$ was absent in both strains (Figure A-2, lanes 5 and 6) this suggests that it is unstable and that its stability is independent of DegP.

A.4 Conclusions.

We showed that a truncated version of CpxP, MBP-CpxP$_{40\text{-}151}$, that is missing regions of both the N-terminus and C-terminus of the protein is stable (Figure A-2) and can still inhibit the Cpx pathway (Table A-2). This suggests that the first 20 amino acids and the last 15 amino acids of the mature CpxP protein are not important for pathway inhibition or stability. While MBP-CpxP$_{20\text{-}151}$ is instable in both a wild type and *degP* null background. Thus, suggesting that MBP-CpxP$_{20\text{-}151}$ is stability may be due to misfolding.

Table A-1. Bacterial strains and plasmids used in Appendix A.

Strains and Plasmids	Description	Reference
Strains		
MC4100	F⁻ *araD139* Δ(*argF-lac*)U169 *rspL150* (Str^R) *relA1 flbB5301 decC1 ptsF25 rbsR*	Casadaban, 1976
TR50	MC4100 λRS88 (*cpxP'-lacZ*$^+$)	Raivio & Silhavy, 1997
TR757	MC4100 λRS88 (*cpxP'-lacZ*$^+$) *degP*::Tn*10*	Buelow & Raivio, 2005
TR783	TR50 (pMCP)	Raivio *et al.*, 1999
DB317	TR50 (pMCP$_{40\text{-}151}$)	This study
DB318	TR757 (pMCP$_{40\text{-}151}$)	This study
DB319	TR50 (pMCP$_{20\text{-}151}$)	This study
DB320	TR757 (pMCP$_{20\text{-}151}$)	This study
Plasmids		
pMal-2p	Cloning vector used to generate periplasmic MBP fusion proteins (Amp^R)	New England Biolab
pMCP	pMal-2p carrying the full length MBP-CpxP fusion protein (Amp^R)	Raivio *et al.*, 1999
pMCP$_{20\text{-}151}$	pMal-2p carrying a truncated MBP-CpxP fusion protein (see Materials and Methods)(Amp^R)	This study
pMCP$_{40\text{-}151}$	pMal-2p carrying a truncated MBP-CpxP fusion protein (see Materials and Methods)(Amp^R)	This study

Table A-2. The effects on Cpx pathway activity when MBP-CpxP$_{20\text{-}151}$ and MBP-CpxP$_{40\text{-}151}$ are overexpressed.

Background[a]	Plasmid	Pathway activity[b]
wild type	pMCP	+
	pMCP$_{40\text{-}151}$	+
	pMCP$_{20\text{-}151}$	-
degP::Tn10	pMCP	+
	pMCP$_{40\text{-}151}$	+
	pMCP$_{20\text{-}151}$	-

a. both wild type (TR50) and *degP*::Tn10 (TR757) background contains a single copy *cpxP'-lacZ*$^+$ transcriptional fusion on the chromosome at the λRS88 site.

b. "+" indicates pathway inhibition, "-" indicates no inhibition of pathway activity.

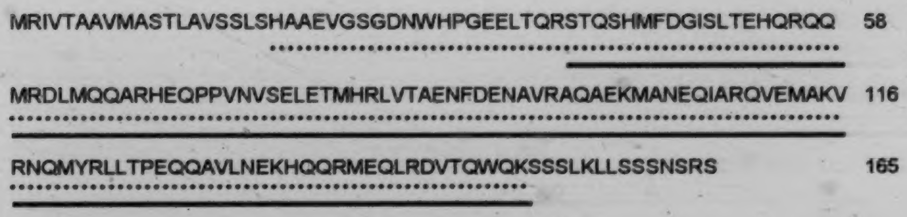
MRIVTAAVMASTLAVSSLSHAAEVGSGDNWHPGEELTQRSTQSHMFDGISLTEHQRQQ 58
MRDLMQQARHEQPPVNVSELETMHRLVTAENFDENAVRAQAEKMANEQIARQVEMAKV 116
RNQMYRLLTPEQQAVLNEKHQQRMEQLRDVTQWQKSSSLKLLSSSNSRS 165

Figure A-1. Sequence and schematic representation of truncated versions of CpxP. Sequence of the full length CpxP is represented here, along with the sequences of the truncated CpxP proteins. CpxP$_{40\text{-}151}$ (13kDa product) is represented by the black line, while CpxP$_{20\text{-}151}$ (15.5 kDa product) is represented by the dotted line.

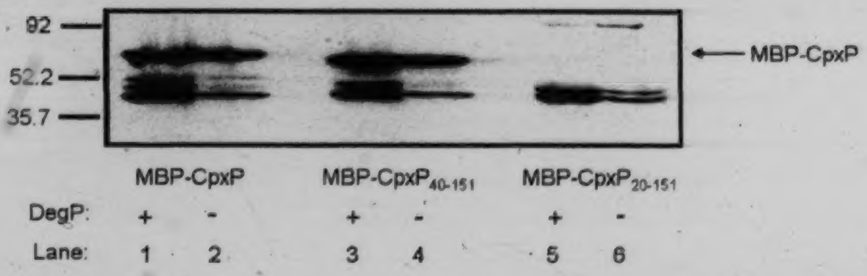
92
52.2
35.7
MBP-CpxP
MBP-CpxP
MBP-CpxP40-151
MBP-CpxP20-151
DegP: + - + - + -
Lane: 1 2 3 4 5 6

Figure A-2. Expression levels of wild type and truncated versions of MBP-CpxP. Western blots were performed on whole cell lysates collected from wild type (odd lanes) and *degP* null (even lanes) strains that were transformed with either pMCP (lanes 1 and 2), pMCP$_{40-151}$ (lanes 3 and 4) or pMCP$_{20-151}$ (lanes 5 and 6). Versions of MBP-CpxP were detected using the polyclonal CpxP antibody.

A.5 References

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